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（プロバイオティクスのヒト大腸ムチンの
酸性基に対する付着性研究）

I-Nung Huang

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CHAPTER 1

Introduction

The consumption of fermented milk has been associated with beneficial health effects. A century ago, Elie Metchnikoff (a Russian scientist, Nobel laureate) postulated that the foods such as yogurt, kefir, and sour milk containing lactic acid bacteria offered health benefits capable of promoting longevity. He reported that Bulgarian peasants who consumed large quantities of Bulgarian sour milk lived longer (1). Henry Tissier, in parallel with Metchnikoff, first isolating bifidobacteria from a breastfed infant, proposed that bifidobacteria might be effective in preventing infections in infants, due to their predominant existence of the intestinal microflora of breastfed infants (2). The works of Metchnikoff and Tissier were the first to make people concerning the probiotic use of bacteria, even if the word "probiotic" was not yet created. After their findings, the studies of the beneficial effects on the host by the consumption of microorganisms has been started to develop.

Probiotics, the term first introduced by Lilly and Stillwell in 1965, were defined as growth-promoting factors produced by microbes that stimulate the growth of other organisms, in contrast to antibiotics (3). In 1989, probiotics were redefined by Roy Fuller as living bacteria that have beneficial effects on the host by improving the balance of microflora in the intestine (4), which has been broadened to state that "a probiotic is a mono- or mixed culture of live microbes which, when applied to animal or man, affect the host beneficially by improving the properties of the indigenous microflora" (5). However, the definition of probiotics has been changed in these years. In 2001, a joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) expert consultation on health and nutritional properties of powder milk with live lactic acid bacteria redefined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host", pointing the importance of the viability and an adequate dose of

probiotic bacteria in order to exert the desirable effects (6). However, many studies indicated that the dead cells of probiotics also generate beneficial biological responses, while the components of dead cells induce an anti-inflammatory response in the host intestine (7). Since the use of dead probiotics has several attractive advantages (e.g. safety and long shelf-life), redefinition or a new definition would be needed for the coming products of probiotics in the future.

Some desirable characteristics of probiotic microorganisms were described, such as:

1. Human origin
2. Generally recognized as safe (GRAS) status
3. Nonpathogenic and noninflammatory
4. Resistance to low pH and bile salts
5. Adhesion to the host gut and proliferation
6. Survival in association with the mucosal immune system
7. Immunostimulatory for the mucosal immune system
8. Technological suitability and so on (8).

Nowadays, probiotics are of significant importance to food and health industries due to their health-promoting effects when consumed. Species of *Lactobacillus* and *Bifidobacterium* are most commonly used as probiotics. Studies have documented probiotic effects on dysbiosis (9), acute diarrhea (10), inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), colon cancer (11), vaginal infection (12), allergy (13), and eradication of *Helicobacter pylori* (14). For the purpose of being sufficient host-interaction to confer health benefits, adhesion ability to the host gut has been considered as one of the selection criteria for probiotic strains. After oral

consumption, probiotics are believed to temporarily colonize the host intestine by adhering to intestinal surface.

The gastrointestinal (GI) epithelium is always exposed to digestive enzymes, fecal material, microorganisms and their products in lumen. There is a layer of mucus lining on the GI tract, which plays an important role in lubricating and protecting the epithelium from mechanical and chemical stress. The mucus layer is the first line against luminal substances, especially the commensal bacteria and invading pathogens, providing a physical and semi-permeable barrier between the epithelium and the contents of lumen. The thickness of the layer is around 400 μm in the intestine (15), which contains 2 layers (16). The thinner inner mucus layer is difficult to dislodge, whereas the outer layer mainly consists of secreted mucins, nonspecific antimicrobials and specific antimicrobial immunoglobulins (17).

Mucins, the major protein components of the mucus, are glycoproteins with high molecular weight from 0.5×10^6 Daltons to 25×10^6 Daltons. The secretion is mainly by goblet cell but also by epithelium cell in the GI tract (18). The formation of mucin starts in endoplasmic reticulum with the production of the protein backbones, which comprises tandem repeats rich in threonine, serine and/or proline residues. After that, the oligosaccharides are highly attached to the variable number tandem repeats (VNTR) of the backbones by O-linked glycosylation range from 1 to more than 20 residues in the Golgi apparatus (Fig. 1-1). Moreover, *N*-acetylneuraminic acid (Neu5Ac, the predominant sialic acid found in mammalian cells) and sulfate residues are added to the end of the oligosaccharides. The oligosaccharide structures can be linear or branched, and acidic or neutral in nature, which shows a huge variation in different parts of GI tract (19, 20).

Based on different protein cores encoded from human genome, mucin could be divided into over 20 types (19). In human GI tract, there are about 7 kinds of mucin existed, and MUC2 mucin is the predominant secretory and gel-forming mucin, which is specifically secreted by goblet cells (Table 1-1). MUC2 gene has been mapped to chromosome 11p15, and is a highly repetitive gene (21). In the tandem repeat domain, approximately 78% of the threonine is glycosylated, which involved in its gel-forming characteristic. After exocytosis, MUC2 mucin may bind to each other and become oligomer via disulfide bond (22). Additionally, the human genome encodes at least four gel-forming mucins, but MUC2 is the only gel-forming mucin and a major component in the mucus of human intestine, and only expressed in goblet cells in intestine (23).

The carbohydrate part of mucin can constitute at least 70% of the total mass and provide many necessary properties, such as protease resistance and water-holding capacity (24). The sugar chains of mucin are attached to the core protein via an O-glycosidic linkage between a serine (or threonine) residue and an *N*-acetyl-galactosamine (GalNAc) residue (25). Despite the heterogeneity of the mucin sugar chain is extremely large, there are main structural features of O-glycan found in intestinal mucin. The sugar chains can be divided into 3 separate regions, which are core structure, type chain and terminal structure (Fig. 1-2). There are over 8 types of core structure have been found in human, but only 5 expressed in the intestine (Table 1-2). The predominant core structure of mucins in every part of the intestine from adults is core 3 (26), whereas the fetal mucin is mainly based on core 2 structures (27). After the repeated type chain, the terminal region is made up of saccharides (e.g. blood group antigen) or acidic residues (Neu5Ac and sulfate) (28).

Along the human intestine, mucin was found to become more and more acidic, and contain less fucose residues. In the ileum, about 30% of mucin oligosaccharides are acidic, but in the distal part more than 65% contain acidic residues (29). According to recent studies, NeuAc residues were mainly α2-6 linked to the GalNAc, and the sialylated core 3 structures were covered in all parts of intestine. On the other hand sulfate was essentially 3-linked to Gal or 6-linked to GlcNAc residues, which sulfated glycans were mainly recovered in distal colonic mucins (26, 29).

The acidomucin in the colon is proposed to protect against enzymatic digestion from high concentrations of bacteria (30). Sulfation of mucin confers resistance to enzymic degradation of the mucus barrier by bacterial glycosidases (31), and sialic acid has been implicated in the protease inhibition by mucins (32). Intestinal infections and the development of experimental colitis using dextran sulfate sodium in mice increased by knocking out the intestinal sulfate transporter; and showed the protective function offered by sulfomucins *in vivo* (33, 34). Some studies indicate an alteration of mucin chemotype usually comes with changes in microflora and intestinal inflammation. For example, the expression of acidomucin was different in IBD where the sulfation of mucins was decreased (35) and the expression of sialomucin was increased (36). The interaction between acidomucin and microflora remains unclear; however, there is a positive correlation between the expressed proportion of sulfomucin and sulfate-reducing bacteria, which can use sulfate from sulfomucin for sulfate respiration in the intestine (37, 38). Other microflora bacteria showed the degradation ability by expression of glycosulfatase specifically for human gastric (39) and oral mucin (40). In addition to a source of nutrients, mucin also serves as binding sites for microbes. Bacteria adhering to the core protein (41), sugar chains, and terminal residues (including blood group antigens, sialic acid and sulfate)

in the mucin structure (42, 43) was reported. However, few studies have been conducted to investigate the binding between microbes and the acidic sugar residues (44-46); and therefore there is a lack of clarity about interactions between probiotics and acidomucins.

Accordingly, for the purpose of investigating the adhesion of probiotics to acidic residues in human colonic mucin, a new evaluation system for screening probiotics with adhesive properties to Neu5Ac and sulfate residues was constructed using the Biacore adhesion assay in the next chapter. After that, the adhesion properties of the selected probiotics were further investigated in chapter 3, and the detection and identification of adhesin-like components with specific affinity to sulfate residue were performed in chapter 4. Since the acidic residues are possible adhesion site for inflammatory pathogens, the results in this study would be expected to provide hints for curing the bowel disease related to altered glycosylation.

“About Biacore”

Biacore is a system for real-time biomolecular interaction analysis. It monitors the formation and dissociation of biomolecular complexes on a sensor surface as the interaction occurs. By covalently attaching one molecule (ligand) to the surface, the interaction of another molecule in solution (analyte) with the ligand is followed. Measurements are made under conditions of continuous flow. For the majority of applications, the biospecific surface can be regenerated and reused for an extended series of analyses.

The measurement in Biacore is performed using surface plasmon resonance (SPR). SPR is a non-invasive optical measuring technique which measures the mass concentration of biomolecules in close proximity to a specially prepared surface (Fig. 1-3). The technique does not require any labeling of the interacting components. The response is essentially independent of the nature of the biomolecule, so that all steps in an interaction analysis may be followed with the same detection technique.

In a Biacore adhesion aasay, the following steps are proformed. A sensorgram is a plot of response against time (Fig. 1-4), showing the progress of the interaction. After the ligand immobilized on a sensor chip, the solution of analyte can be injected to the selected flow cell. When the injection of analyte stops, the buffer from the system will start to flow. The adhesion value should be observed in this step (Fig. 1-5). Finally, by an injection of acidic (glycine-HCl buffer or dilute HCl), basic (NaOH) or surfactant solutions, the bound analyte should be removed from the sensor chip surface without destroying the ligand activity. The process here is called regeneration. The number of times a sensor surface can be regenerated depends on the nature of the attached ligand, but is usually greater than 100 and may even be 1000 or more. Time spent on the adhesion assay would be about 20 min one time. The amount of ligand and analyte is

not constant in every test. For most proteins, concentrations of 10-50 μg/mL are sufficient, and higher concentrations simply consume more ligand without significantly improving the results. Lower concentrations may be used in favorable cases. The analyte concentration is around 1-3 mg/mL in this study.

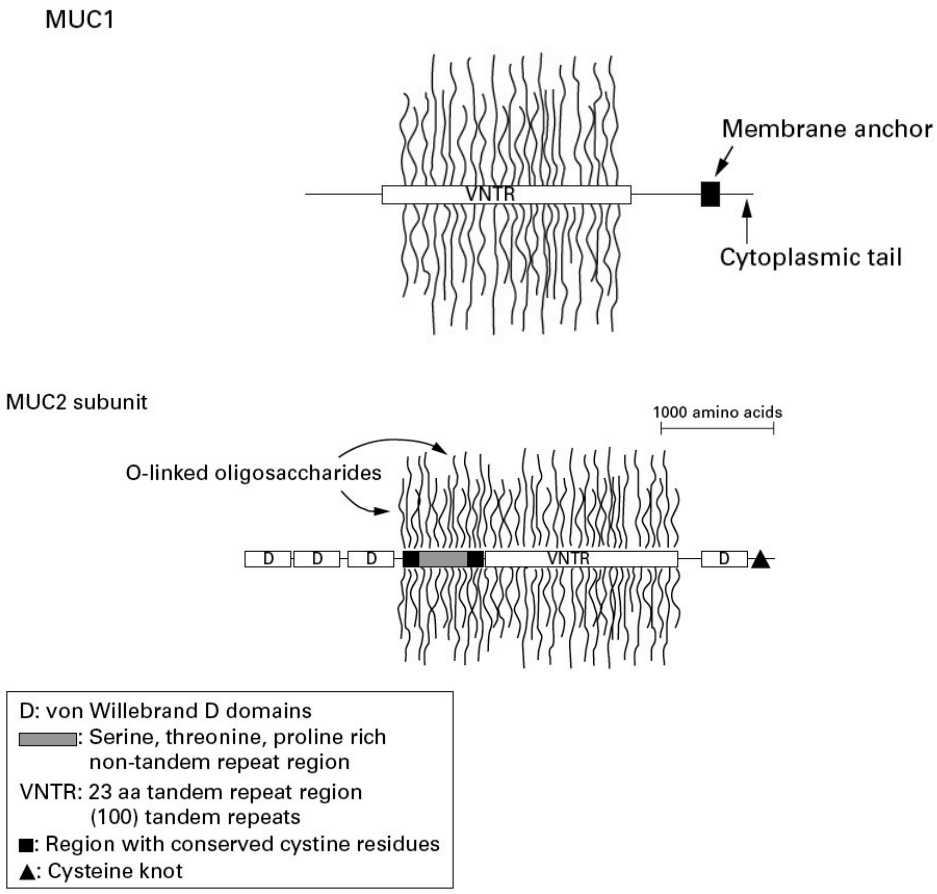


Figure 1-1. The Structure of MUC1 and MUC2 (20).

Table 1-1. Different types of mucin in intestinal tract of human, including large and small intestine (23).

Gene	Type	Expression
MUC1	Membrane-associated	Mammary gland, cervix, pancreas, lung, large intestine
MUC2	Secretory	Small intestine, large intestine, respiratory tract
MUC3	Membrane-associated	Small intestine, large intestine, gallbladder, lung
MUC4	Membrane-associated	Large intestine, stomach, mammary gland, cervix, lung
MUC12	Membrane-associated	Large intestine, pancreas, prostate, cervix
MUC13	Membrane-associated	Large intestine, respiratory tract, kidney, small intestine, stomach
MUC21	Membrane-associated	Lung, thymus, large intestine

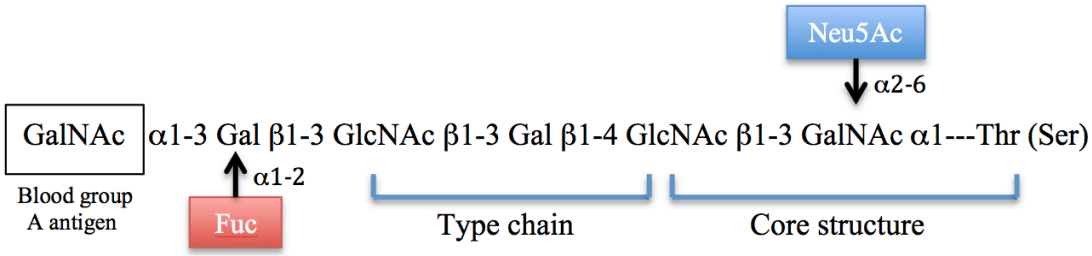


Figure 1-2. The representative structure of the oligosaccharides in intestinal mucin which is based on the core 3 structure with type chain and blood type A antigen (26, 47).

Table 1-2. Oligosaccharide epitopes on intestinal mucin O-glycans (48).

Epitope	Core structures
Core 1	Galβ1-3GalNAc
Core 2	GlcNAcβ1-6 (Galβ1-3) GalNAc
Core 3	GlcNAcβ1-3GalNAc
Core 4	GlcNAcβ1-6 (GlcNAcβ1-3) GalNAc
Core 5	GalNAcα1-3GalNAc
	Type chain
Type 1	-Galβ1-3GlcNAcβ1-
Type 2	-Galβ1-4GlcNAcβ1-
	Terminal structures
Blood group H	Fucα1-2Galβ1-
Blood group A	GalNAcα1-3 (Fucα1-2) Galβ1-
Blood group B	Galα1-3 (Fucα1-2) Galβ1-

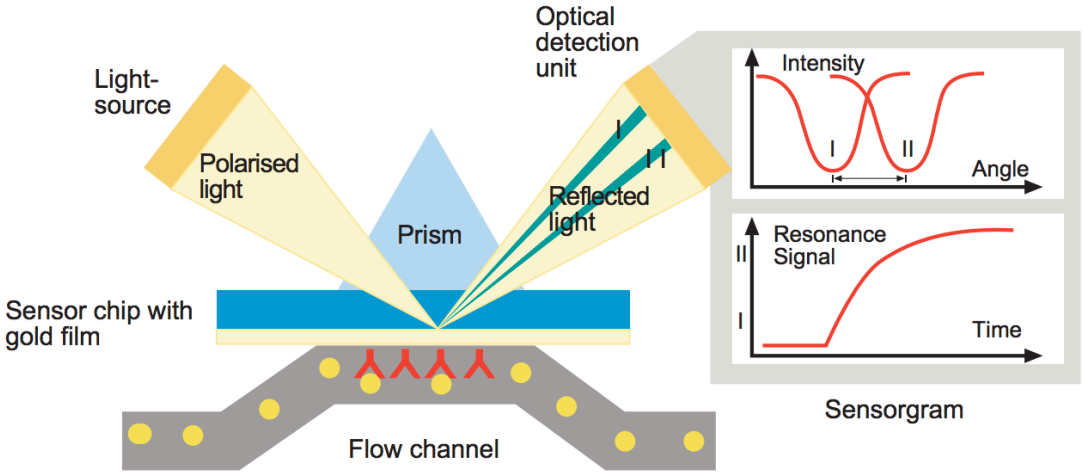


Figure 1-3. A ligand-analyte interaction of Biacore analysis using surface plasmon resonance.

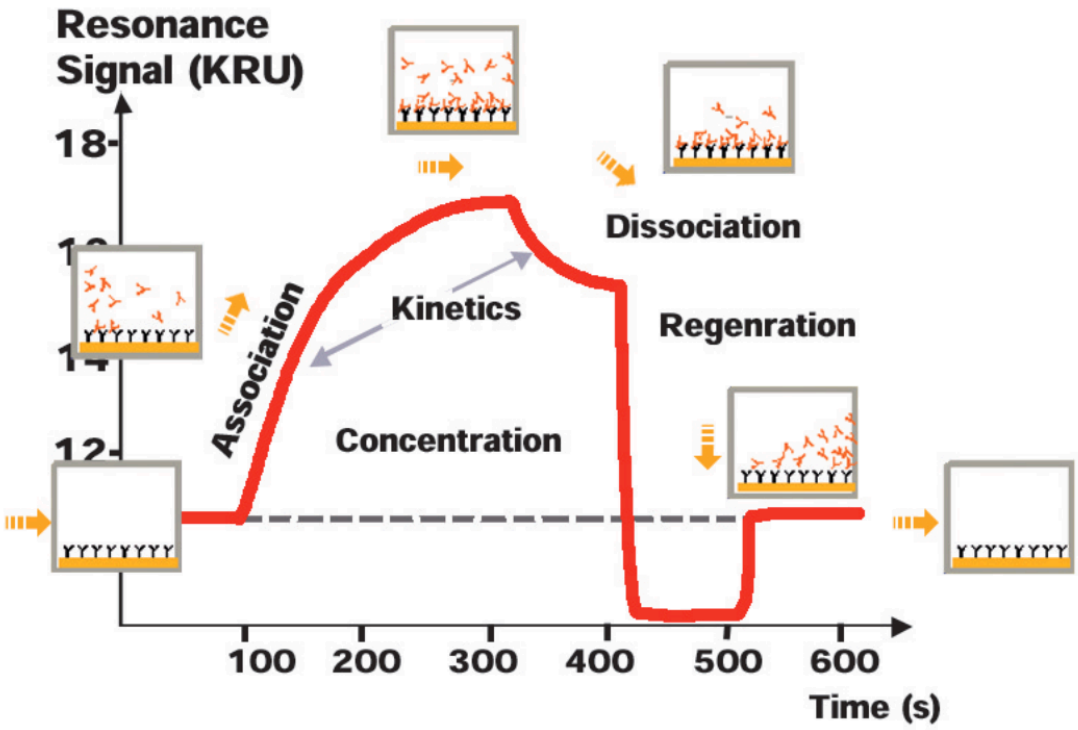


Figure 1-4. A typical sensorgram of Biacore and the interpretation.

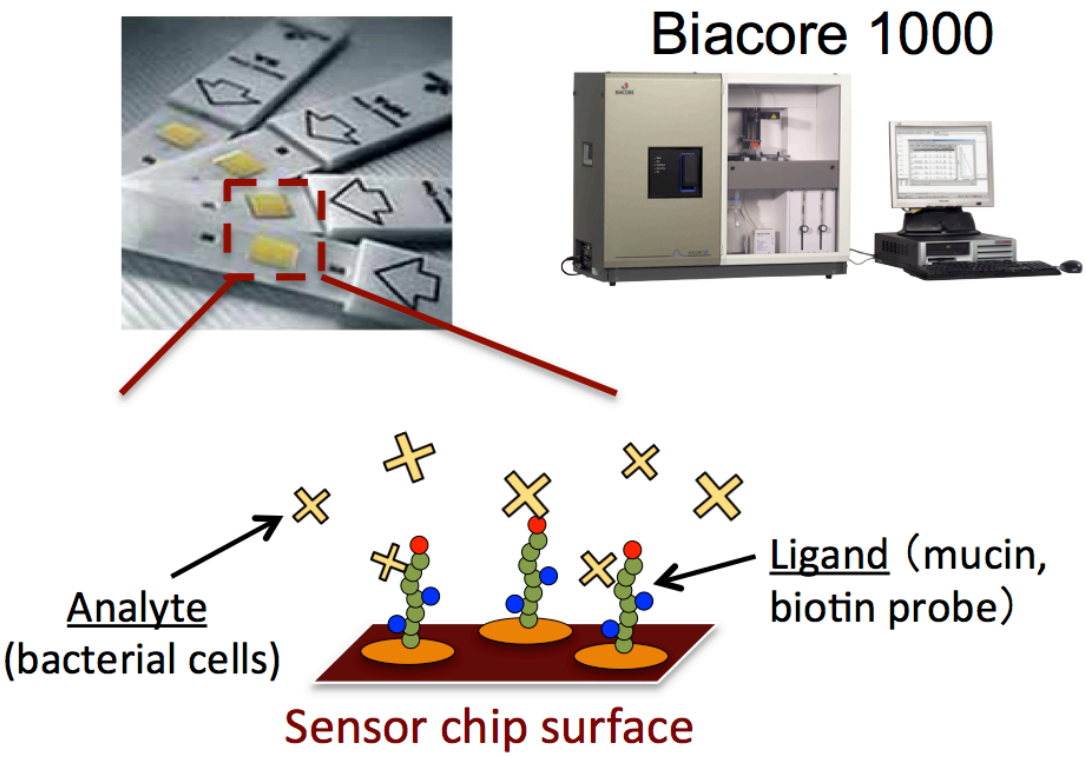


Figure 1-5. Outline of Biacore apparatus

CHAPTER 2

New screening methods for probiotics with adhesion properties to acidic residues in human colonic mucin using the Biacore assay

Introduction

Mucin is the major component of the intestinal mucus layer, and can be divided into neutral and acidic mucin. Acidic mucin is further classified into sialomucin with sialic acid (*N*-acetylneuraminic acid, NeuAc) and sulfomucin with sulfate residues that bind to the sugar chain of mucin. The binding position of these acidic residues is limited in human intestinal mucin where they link to specific positions on the sugar chain. Sialic acid residues expressed in mucin were found primarily α2-6 linked to the initial *N*-acetylgalactosamine (GalNAc) and to a lesser extent α2-3 linked to the terminal galactose (Gal). Sulfate residues were primarily linked to the C-3 of terminal Gal or less so to the C-6 of *N*-acetylglucosamine (GlcNAc) (Fig. 2-1, based on Robbe *et al*.) (29). The expression of sialomucin and sulfomucin increases gradually along the human colon where sulfomucin is predominant (38). Since increased bacterial concentrations were also observed in descending colon, the acidic residues are proposed to protect colon mucin against enzymatic digestion from microflora (30).

Few papers report the interaction between acidomucin and microflora; some assays were used in measuring the binding between them. The most commonly used method is the microplate assay. After immobilization of mucin on the plate, enzymatic cleavage or chemical treatment can be performed before adding target bacteria or the surface component (49). Conversely, when bacteria or the surface component is immobilized on the plate, adding desialylated or desulfated mucin to measure the remaining adhesion was investigated (45). Immunodetection by dot blot analysis was used. Using desialylation or desulfation of biotinylated mucin, the binding of bacterial components to acidomucin was observed qualitatively (46). However, the microplate assay is a quantitative and efficient method requiring many artificial operations that

may introduce error in the experiment. A sensitive, quantitative analysis method with fewer artificial operations was required. Therefore, a modified binding assay using Biacore was developed for evaluating the adhesiveness of lactobacilli and bifidobacteria to sialic acid and sulfate residues of mucin.

In the previous studies using the Biacore adhesion assay, several *Lactobacillus* species isolated from the human intestine was found to bind to the human blood group A and B antigens of human colonic mucin (HCM). Furthermore, the adhesins from lactobacilli, e.g. the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ATP-binding cassette (ABC) transporter expressed on the surface of bacteria were identified and characterized (43, 50). Using chemical and enzymatic modification of mucin with the Biacore adhesion assay, here I describe a new screening method to evaluate the specific adhesion of probiotics for sialic acid and sulfate residues in intestinal mucin. The removal of sialic acid and sulfate residues were performed using sialidase and sulfatase, respectively. Blockage of sulfate was performed by elution with barium chloride using the Biacore assay. Additionally, some improvements in the HCM purification procedure were made. Because there is a relationship between the expression of mucin chemotypes, microflora and intestinal inflammation, building an evaluation system to clarify the binding of acidomucin and microorganisms may be helpful to understand the adhesion strategies of bowel disease pathogens and microflora in the digestive tract.

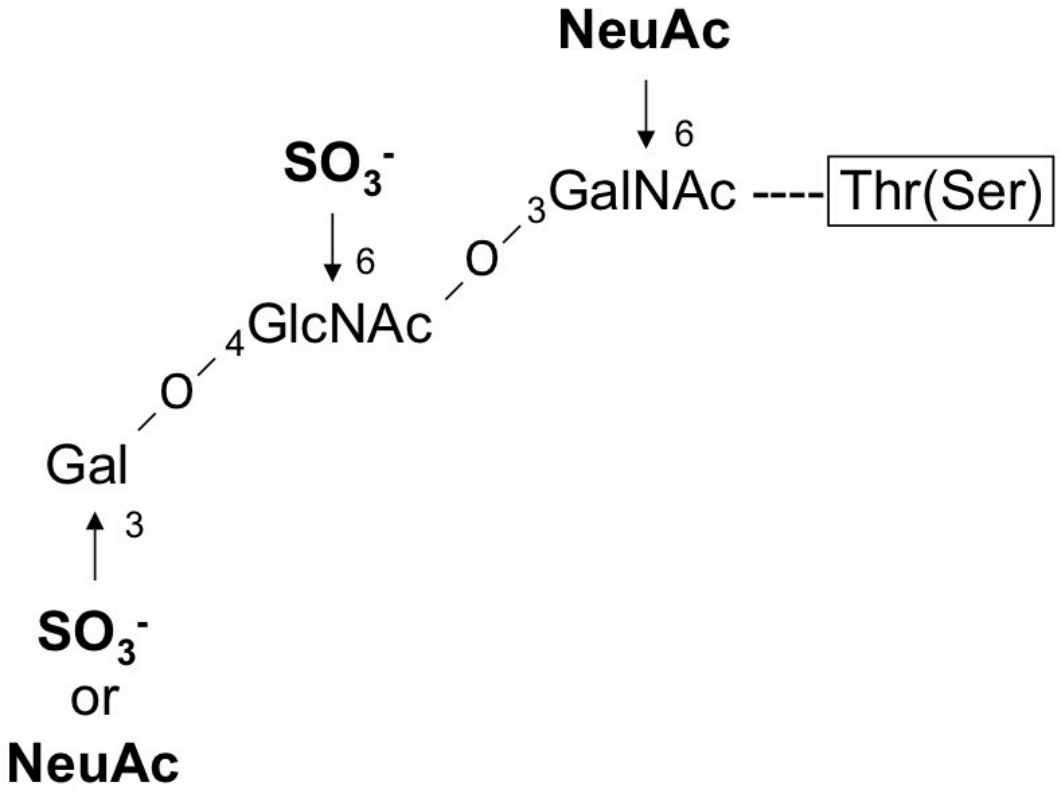


Figure 2-1. Representative binding position of N-acetylneuraminic acid (NeuAc) and sulfate (SO3-) on the sugar chain of human colonic mucin (based on Robbe *et al*. 2004). Since the sugar chain expressed in human intestinal mucin could be shorter or longer with different compositions, this figure only shows the possible binding sites of the acidic residues.

Materials and Methods

1. Bacterial strains and culture

Ten strains of lactobacilli and three strains of bifidobacteria were used for the adhesion assay in this study (Table 2-1). Lactobacilli were provided from the culture collection of Meiji Co., Ltd. (Tokyo, Japan); and bifidobacteria was a gift from Morinaga Milk Industry Co., Ltd. (Zama, Japan). All strains were isolated from human feces. Lactobacilli strains were grown in Man Rogosa Sharpe (MRS) broth (Difco Laboratories, Detroit, MI); and propagated twice at 37 °C for 16 h with a 2% (v/v) inoculum. Bifidobacteria strains were cultured anaerobically in GAM broth (Nissui Seiyaku, Tokyo, Japan) twice at 37 °C for 24 h with a 10% (v/v) inoculum. Bacterial cells were collected from the culture medium using centrifugation (8500 × ***g***, 4 °C, 10 min) and washed three times with phosphate-buffered saline (PBS, pH 7.4). After removing the media components, the bacterial cells were lyophilized. Before using the Biacore assay, the cells were suspended in HBS-EP buffer (HEPES-buffered saline with EDTA and polysorbate 20: 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20; GE Healthcare Bio-Sciences K.K., Tokyo, Japan) as the analyte (3 mg/mL) in the Biacore assay.

2. Purification of soluble human colonic mucin

This study was approved by the ethics committee of Tohoku University Graduate School of Medicine. Human colon mucus was obtained from Tohoku University Graduate School of Medicine. Crude mucus was scraped from the normal intestinal tissues of colorectal cancer patients; and extracted with chloroform: methanol (2:1, v/v) and diethyl ether three times respectively to remove lipid. The lipid-free HCM

was digested with 0.5 mg/mL Proteinase K (TaKaRa Biotechnology, Shiga, Japan) overnight. After centrifugation (8500 × ***g***, 4 °C, 10 min) and membrane filtration (DISMIC-25, 0.45 μm, Advantec, Tokyo, Japan), the supernatant was purified by gel filtration chromatography with a Toyopearl HW-65F column (90 × 2.6 cm; Tosoh, Tokyo, Japan) using distilled water as the mobile phase. Peptide was detected at 214 nm and neutral sugar was measured at 490 nm using the phenol-sulfuric acid method. Fractions containing high concentrations of sugars and peptides were collected and concentrated before lyophilization. The purified soluble HCM (sHCM) was used as the ligand for the Biacore analysis.

3. Immobilization of sHCM

All Biacore experiments were performed using a Biacore 1000 (GE Healthcare Bio-Sciences K.K.) at 25 °C in HBS-EP buffer. The immobilization of sHCM on a CM5 sensor chip (GE Healthcare Bio-Sciences K.K.) was an amine coupling reaction following the manufacturer's instructions. sHCM was dissolved at 10 mg/mL in 10 mM sodium acetate buffer (pH 4.0) and immobilized using the reaction between N-hydroxysuccinimide (NHS) -esters and radicals of primary amino groups in sHCM. The sensor chip was equilibrated in HBS-EP buffer.

4. Removal of sialic acid from sHCM

The removal method of sialic acid was the modified van der Merwe *et al*. method (51). After immobilization onto Biacore CM5 sensor chips, sHCM was desialylated by injection of 100 mU/mL *Streptococcus* Neuraminidase F (Seikagaku Co., Tokyo, Japan), enzymatically removing α2-3, α2-6, and α2-8 linked sialic acid (used as sialidase in this study) in 10 mM citrate phosphate buffer (pH 6.5) with 2 mM $CaCl_2$

at a flow rate of 1 μL/min at 25 °C for 12.5 h; and then equilibrated in HBS-EP buffer (Fig. 2-2).

5. Removal and blocking of sulfate groups in sHCM

The desulfation method was modified from Fong *et al*. (52) and performed in test tubes. sHCM was treated with 2 U/mL *Helix pomatia* sulfatase (Sigma-Aldrich, MO, USA) in 100 mM sodium acetate buffer (pH 5.0) overnight at 37 °C using a sham digestion control with boiled enzyme. The enzymatic reaction was terminated by boiling for 15 min. After dialysis of treated sHCM against 10 mM sodium acetate buffer (pH 4.0) for 6 h at 4 °C, the dialysates were immobilized onto Biacore CM5 sensor chips following the manufacturer's instructions. Further, the mucin-binding sulfate residues on the sensor chip-bound sHCM were blocked using injection of 0.5 M barium chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at a flow rate of 1 μL/min for 12.5 h; and then equilibrated in HBS-EP buffer (Fig. 2-2).

6. Probiotics and lectin adhesion analysis using Biacore

Quantifying adhesion using the Biacore 1000 is based on the principle of surface plasmon resonance (53). After washing and lyophilization, bacterial cells were suspended at a concentration of 3 mg/mL in HBS-EP buffer. After injecting the bacteria suspension at a flow rate of 3 μL/min for 5 min, the sensor chip was washed with HBS-EP buffer to remove unbound analyte; and regenerated eluting with 1 M guanidine hydrochloride (GHCl) solution at a flow rate of 3 μL/min for 2 min. The resonance units (RU) were measured for 200 s after the cessation of sample addition.

For quantifying the adhesion of lectins to sHCM, *Erythrina cristagalli* Agglutinin (ECA, J-OIL MILLS, Inc., Tokyo, Japan) and *Arachis hypogaea* (peanut) Agglutinin

(PNA, J-OIL MILLS) were dissolved at 1 mg/mL in HBS-EP buffer and injected to CM5 sensor chip at a flow rate of 3 μL/min for 15 min. The measurement of RU and other experimental steps were performed following the steps in bacteria adhesion assay above. A response of 1 RU represents about 1 pg/mm^2 protein adhering with increased concentration of analyte bound to the sensor chip surface.

7. Statistical analysis

All experiments were performed in triplicate and reported as the mean ± SD. For adhesion test between sHCM and bacterial cells using the Biacore assay, statistical analysis of the data was performed using one-way analysis of variance (ANOVA test) followed by the *post hoc* Dunnet test when the F value showed significant differences at $p < 0.05$. For adhesion tests between desulfated (or desialylated) sHCM and bacterial cells using the Biacore assay, statistical analysis of the data was performed using the Student's *t*-test. For each statistic, significant differences were inferred whenever the *p*-value was <0.05.

Table 2-1. Lactobacilli and bifidobacteria used in this study

Bacterial species	Strain	Source
Lactobacillus gasseri	OLL2716	Human feces
Lactobacillus brevis	OLL2772	Human feces
Lactobacillus brevis	OLL2775	Human feces
Unidentified *Lactobacillus* species	OLL2785	Human feces
Lactobacillus gasseri	OLL2793	Human feces
Lactobacillus gasseri	OLL2804	Human feces
Lactobacillus gasseri	OLL2827	Human feces
Lactobacillus mucosae	OLL2855	Human feces
Lactobacillus gasseri	OLL2877	Human feces
Lactobacillus mucosae	OLL2882	Human feces
Bifidobacterium breve	MCC167	Infant feces
Bifidobacterium bifidum	MCC1092	Adult feces
Bifidobacterium infantis	ATCC15697	Infant feces

The strains were identified through API 50CHL analytical system and 16S rRNA analysis.

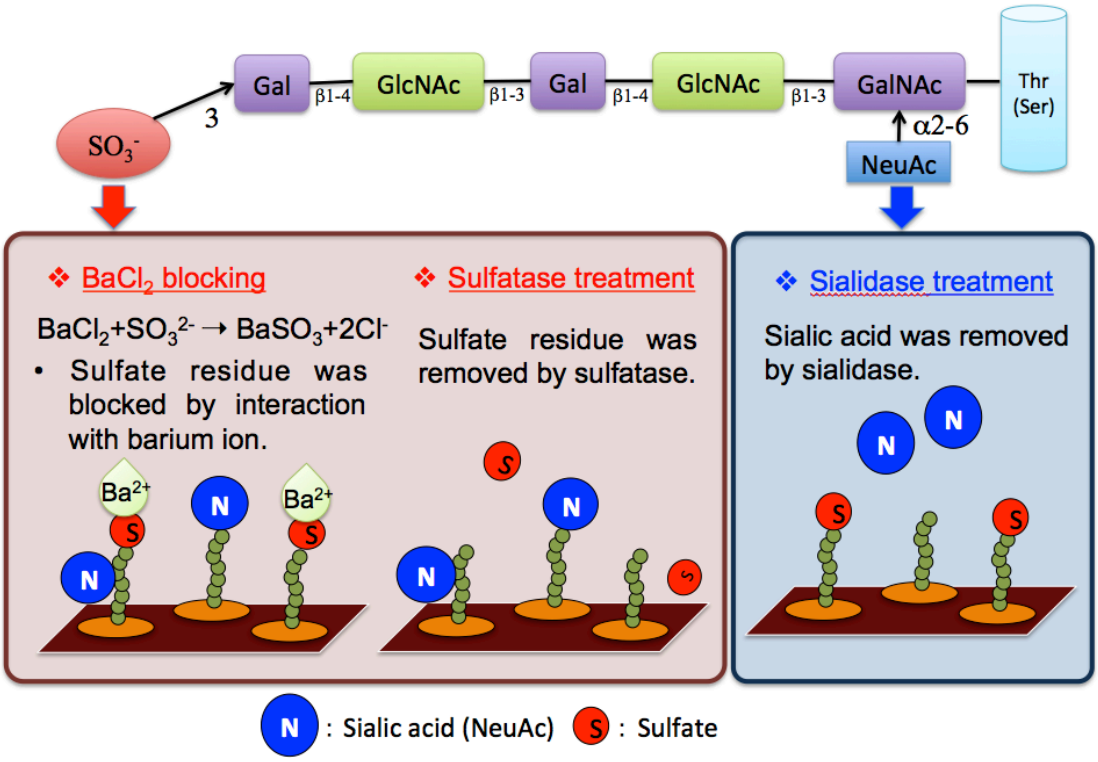


Figure 2-2. The illustration of sHCM treated with sialidase, sulfatase, and barium chloride.

Results

1. Purification of soluble human colonic mucin

In the previous study (54), the de-lipid mucus was dissolved in 4 M GHCl, fractionated it using gel filtration chromatography and collected the fractions containing high sugar and protein concentrations to obtain the purified human colonic mucin (HCM). However, there were some proteins (e.g. human histone H3) found in the HCM that have binding activities for probiotics (55). To analyze the adhesion between the mucin sugar chain and probiotics, the mucin was further purified with Proteinase K to digest these proteins and the core protein structure of mucin. After gel filtration chromatography, the fractions containing high concentrations of sugars and peptides (fraction no. 24 to 84) were collected and lyophilized (Fig. 2-2).

This allowed me to study the adhesion of the mucin oligosaccharides and the terminal residues. Due to its water-soluble properties, I called this soluble HCM (sHCM).

2. Screening of probiotics binding sHCM

Previously, some lactobacilli and bifidobacteria were found to have strong adhesion ability to HCM and human blood group antigens expressed in intestinal mucin (54, 56). Here I used 10 strains of lactobacilli and three strains of bifidobacteria that adhered to HCM strongly; and were selected to measure the adhesive activities to sHCM. Using the Biacore analysis, lyophilized bacterial cells as analytes were analyzed to determine their adhesion abilities to the immobilized sHCM as ligands. Results show there were three probiotics showing significant higher adhesion than others ($p < 0.01$) that were all under 60 RU. *Lactobacillus gasseri* OLL2877 showed the highest adherence (401.2 ± 78.6 RU) to the sHCM and the

adhesion activities of *Lactobacillus* strain OLL2785 and *Bifidobacterium bifidum* MCC1092 strains were 225.5 ± 90.5 RU and 196.3 ± 40.4 RU, respectively (Fig. 2-4). Thus, the three bacterial strains were selected to investigate further the adhesion mechanism of human colonic acidomucin.

3. Probiotics recognize sialic acid on sHCM

After screening the probiotics with higher adhesion activities to sHCM, I analyzed whether the probiotics bound to sialic acid or sulfate residues of mucin oligosaccharides. Using Biacore analysis, sHCM was immobilized onto a Biacore sensor chip CM5 and the sialic acid expressed in sHCM was removed by elution with sialidase overnight on the Biacore biosensor. With sialidase, the α2-3 and α2-6 linked sialic acid binding to sHCM was cleaved, and the peripheral galactose and the initial GalNAc would presumably be exposed. Therefore, the binding value for PNA lectin for sHCM increased significantly after sialidase treatment ($p < 0.001$), since PNA lectin recognizes and binds to the carbohydrate sequence Galβ1-3GalNAc (Fig. 2-5).

After sialidase treatment, the resonance units of the three selected bacteria strains were decreased significantly ($p < 0.05$). The adhesion of OLL2785 strain showed a 7.4-fold decrease, the highest among the three probiotics (Fig. 2-6). This showed the sialic acid expressed in sHCM could be recognized by the three selected bacteria strains and may serve as binding site for the microbes in the intestine.

4. Probiotics recognize sulfate residue on sHCM

The effects of removing sulfate residues on probiotic adhesion were determined. I tried in a similar way to remove sulfate on sHCM-immobilized CM5 sensor chips by elution of sulfatase overnight to remove the 3-linked or 6-linked sulfate residues

expressed in sHCM and disclose galactose or GlcNAc. However, I found the resonance unit of the sensor chip increased after the elution of sulfatase overnight, indicating the sulfatase bound to sHCM. To avoid the effects of binding sulfatase, sham digestions were performed with boiled inactive enzyme. The adhesive assay between probiotics and sulfatase was also performed, and the three probiotics showed a very low binding activity to sulfatase (under 5 RU). Compared to sham digestion groups, the adhesion abilities of three probiotics were different after sulfatase treatment. *L. gasseri* OLL2877 showed a significant decrease after removing sulfate residues in sHCM. However, the adhesion of OLL2785 strain and *B. bifidum* MCC1092 increased ($p < 0.05$) (Fig. 2-7).

With sulfatase, the sulfate residue linked to the C-3 of Gal and the C-6 of GlcNAc was cleaved, and the terminal Gal and the GlcNAc should presumably be exposed. PNA and ECA lectin recognizes and binds to the carbohydrate sequence D-Gal and Galβ1-4GlcNAc, respectively, so I used them to confirm the treatment of sulfatase. However, the binding value for the lectins for sHCM decreased significantly after sulfatase treatment ($p < 0.05$) (Fig. 2-8).

Further, the sHCM-immobilized sensor chip was treated with barium chloride to block the electric charge of the sulfate, a possible binding site for microbes. There was a significant decrease in adhesion after barium chloride treatment ($p < 0.05$). The most significant was OLL2785 strain that showed a 10.4-fold decrease ($p < 0.01$) (Fig. 2-9).

Therefore, only *L. gasseri* OLL2877 showed significant decrease of adhesion after sialidase, sulfatase or barium chloride treatment on mucin; whereas the adherence of OLL2785 strain and *B. bifidum* MCC1092 decreased after sialidase and barium chloride treatment and increased after sulfatase digestion.

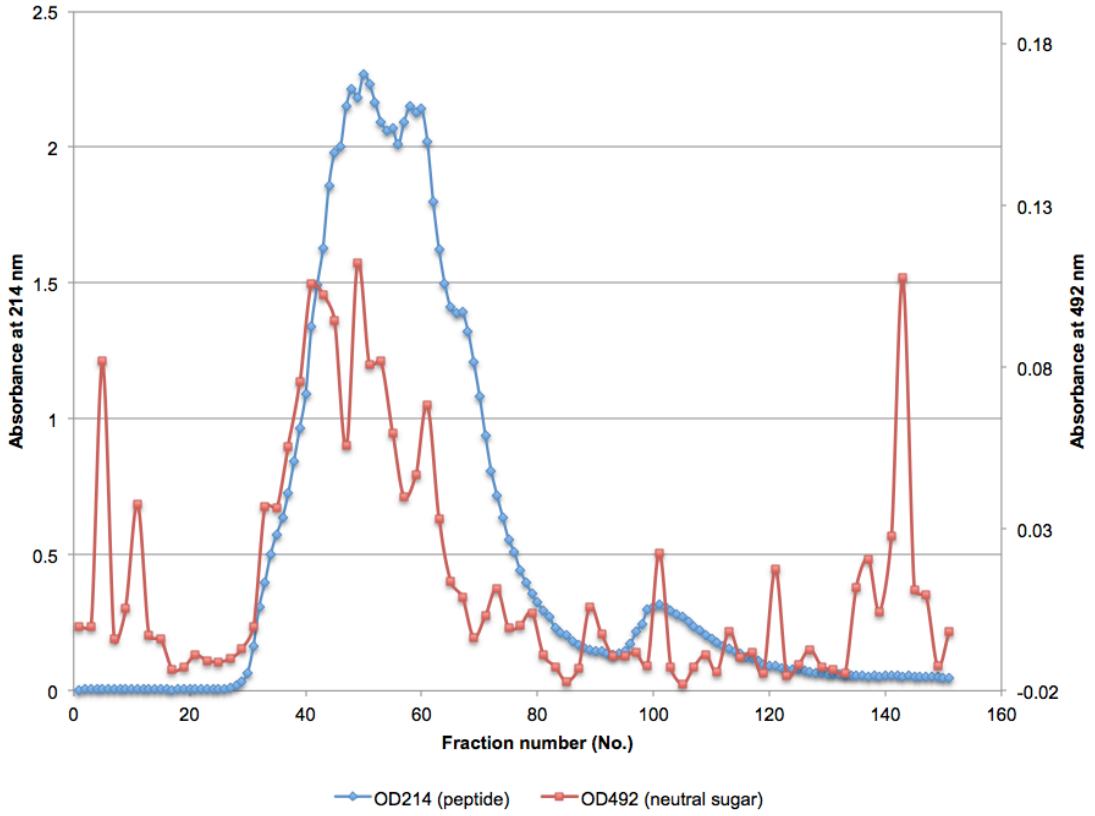


Figure 2-3. The purification of soluble human colonic mucin (type A) by gel filtration chromatography was detected by UV absorption at 214 nm (peptide) and phenol-sulfuric acid method at 492 nm (neutral sugar).

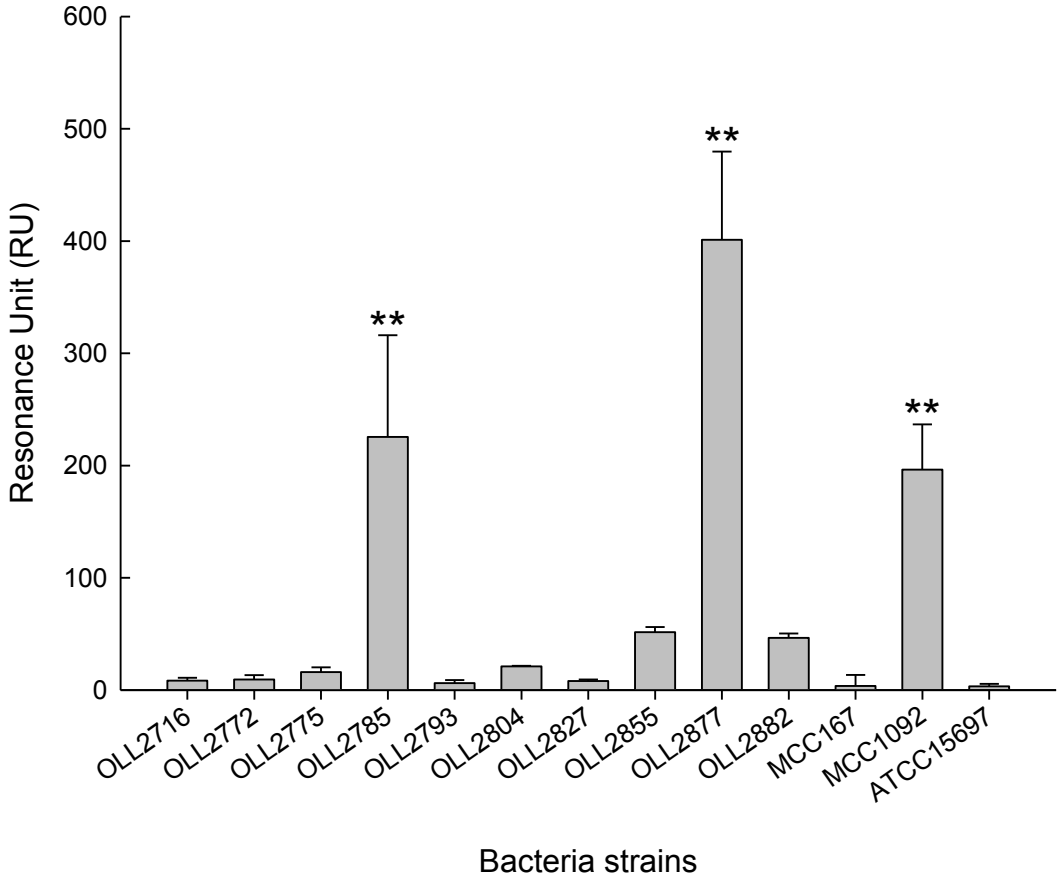


Figure 2-4. Binding abilities of the cell bodies of lactobacilli and bifidobacteria isolated from human feces to sHCM using Biacore analysis. Columns represent the mean ± SD (n = 3). **$p < 0.01$.

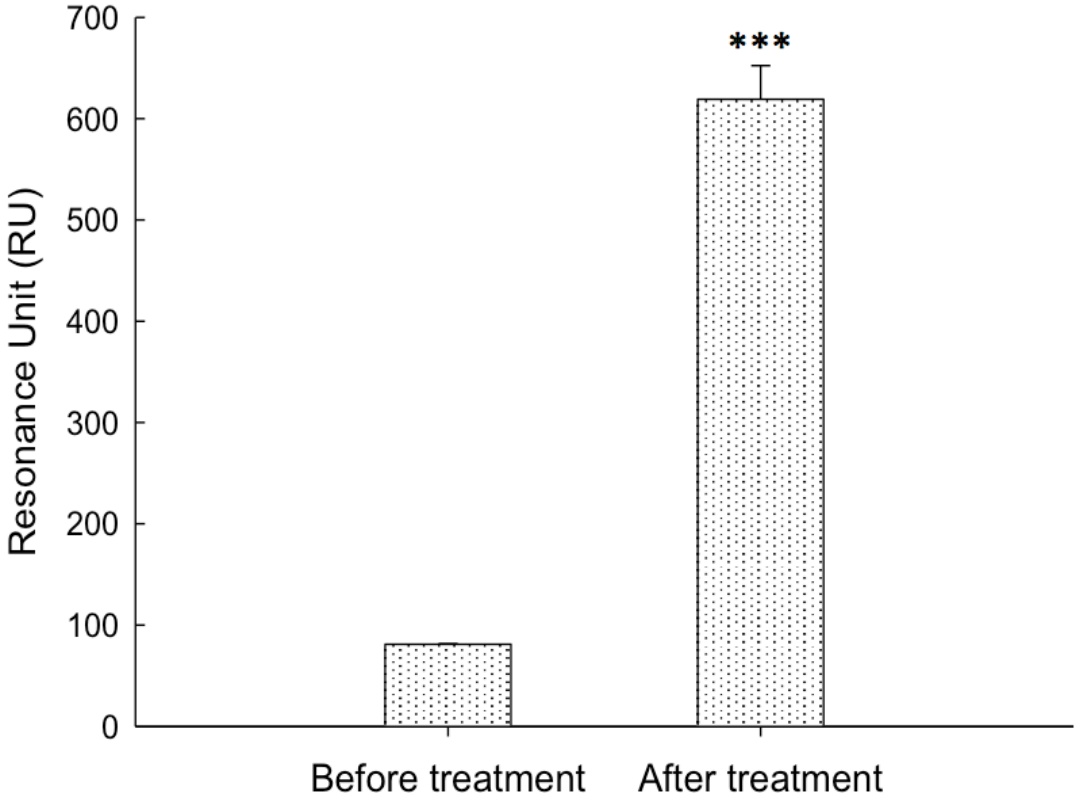


Figure 2-5. The change in binding values for PNA lectin before and after sHCM digested by sialidase. Columns represent the mean ± SD (n = 3). ***$p < 0.001$.

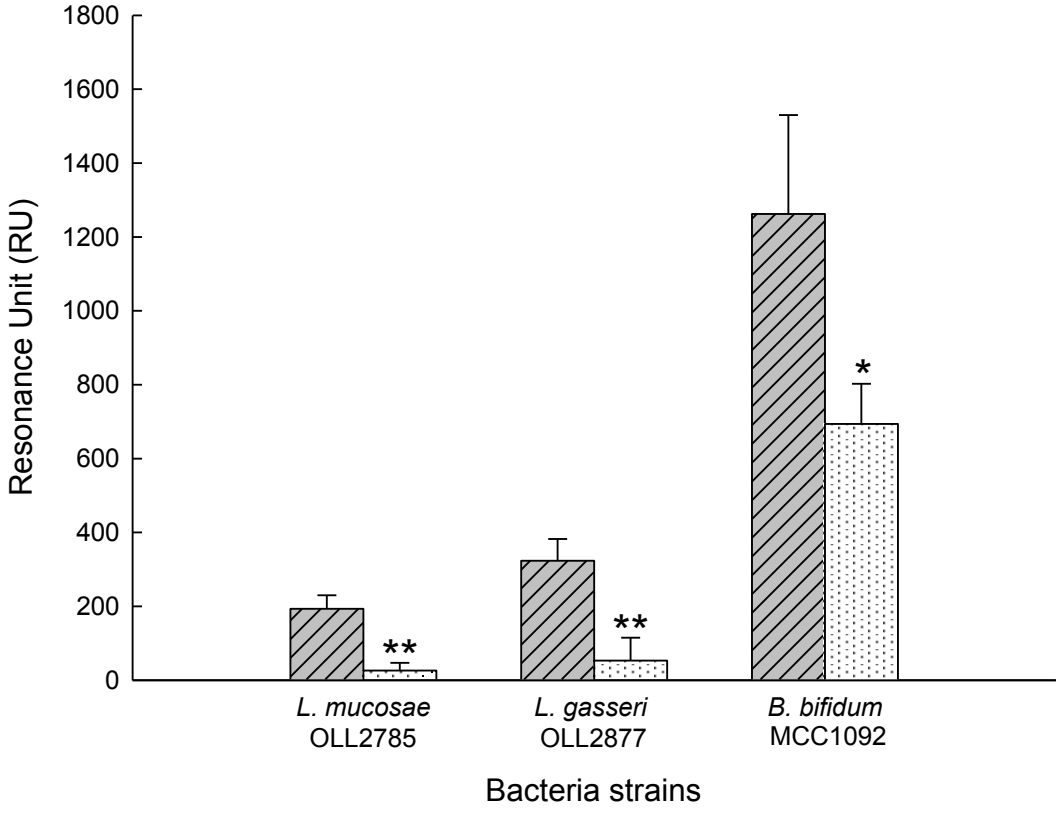


Figure 2-6. The change in adhesion values for the selected three strains for the sugar chain of sHCM before and after sialidase digestion. Columns represent the mean ± SD (n = 3). (▨) before sialidase treatment; (▩) after sialidase treatment. *$p < 0.05$; **$p < 0.01$.

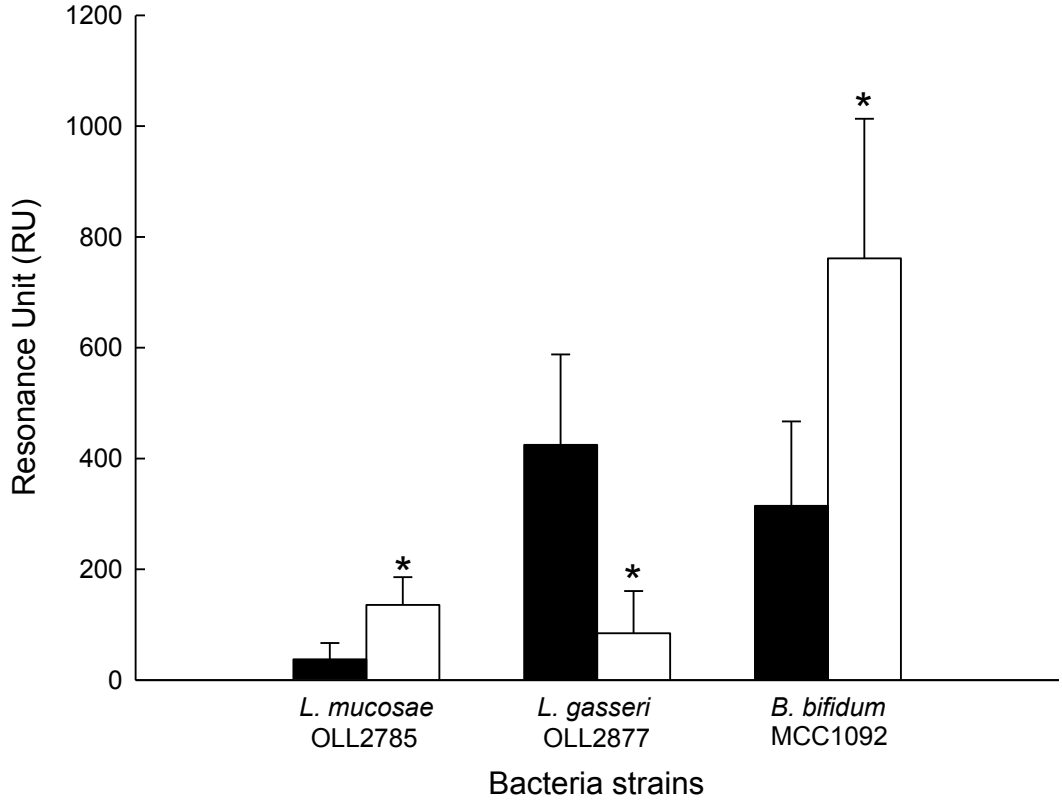


Figure 2-7. The change in adhesion values for the selected three strains for the sugar chain of sHCM before and after sulfatase digestion. Columns represent the mean ± SD (n = 3). (■) adhesion to sham digestion; (□) adhesion to sulfatase digestion. *$p < 0.05$.

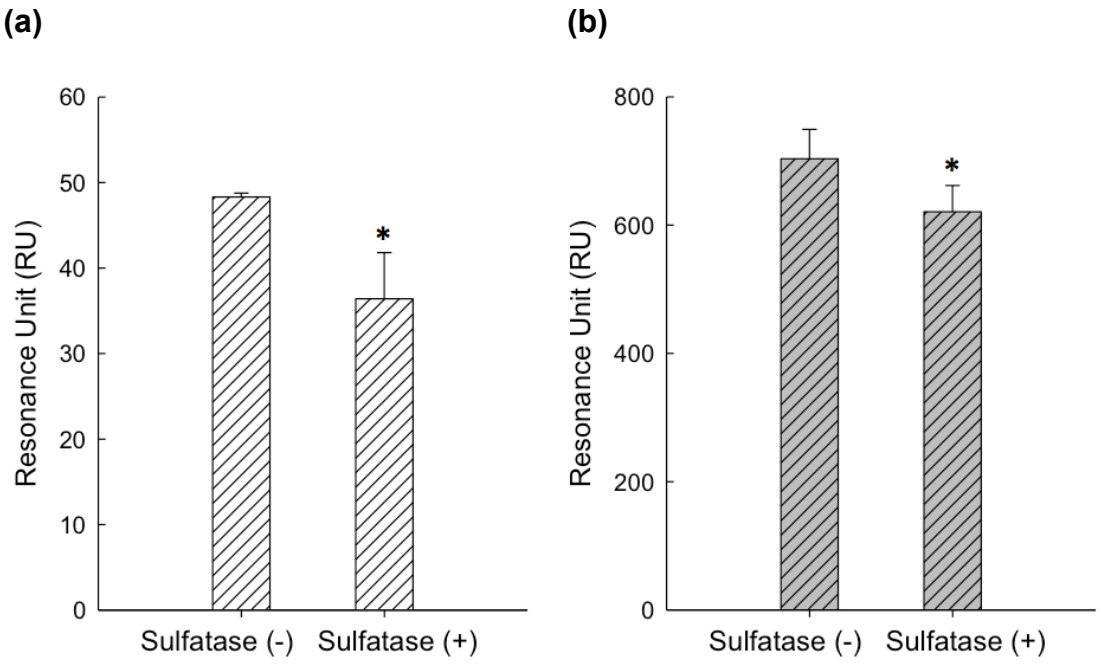


Figure 2-8. The change in binding values for (a) PNA and (b) ECA lectin before and after sHCM digested by sulfatase. Columns represent the mean ± SD (n = 3). *$p < 0.05$.

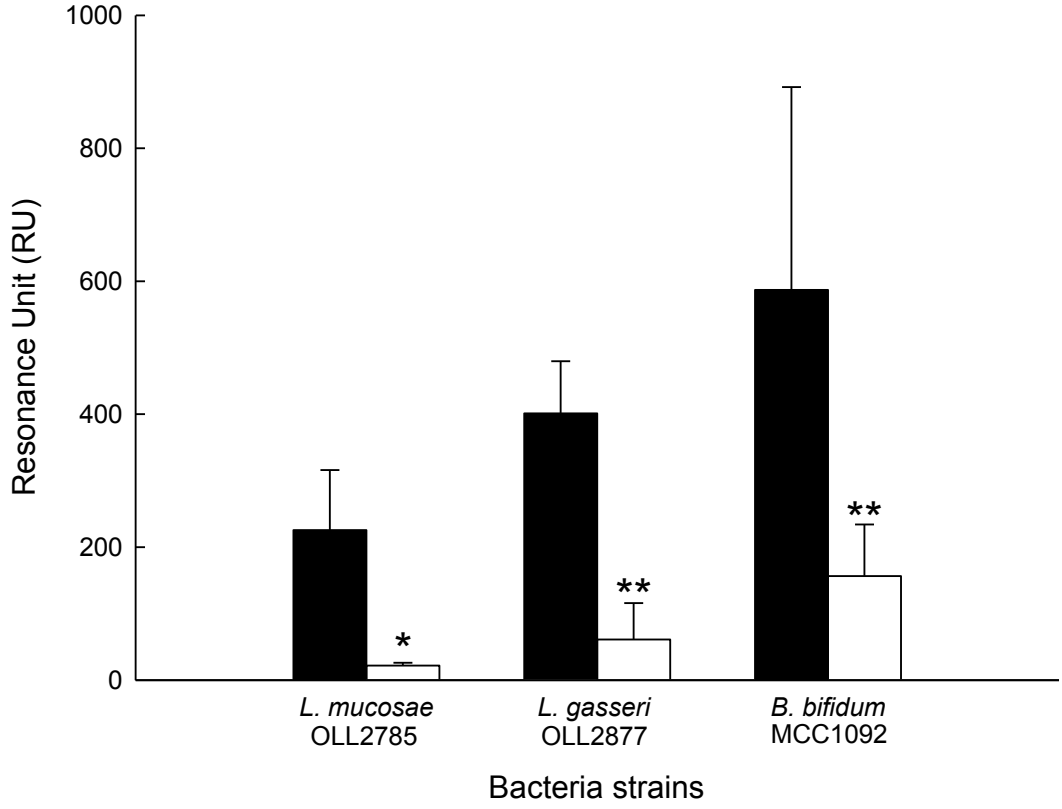


Figure 2-9. The change in adhesion values for the selected three strains for the sugar chain of sHCM before and after $BaCl_2$ treatment. Columns represent the mean ± SD (n = 3). (■) before $BaCl_2$ treatment; (□) after $BaCl_2$ treatment. *$p < 0.05$; **$p < 0.01$.

Discussion

Here, I chose 10 strains of lactobacilli and three strains of bifidobacteria that highly bound to HCM to measure the adhesion to sHCM. However, only two lactobacilli strains and one bifidobacteria strain adhered to sHCM significantly suggesting the other ones bound to the core proteins of colonic mucin or the low-molecular-weight proteins (e.g. histone H3) contained in the previously purified HCM. The adhesin Lam29 isolated from *L. mucosae* was demonstrated to contain specific affinity to human histone H3 from intestinal mucosa (55), and MUC2 was reported as a major chemo-attractant and binding target for *Campylobacter jejuni* in the host intestinal tract (41). These studies and the results in this study suggest the adhesion strategy of microflora is not constant but variable.

Here, a sialidase treatment was performed using the Biacore assay; and the adhesion of three probiotic strains was decreased significantly after removing sialic acid from colonic mucin, suggesting the sialic acid residue was recognized as a binding site by these probiotics. There are some microflora and pathogens also reported to show adherence to sialic acid expressed in mucin. *Helicobacter pylori* is well known to contain a sialic acid-binding adhesin (Sab A) that adheres to sialylated glycoprotein during inflammation (57). Streptococci (45) and *Escherichia coli* (58) were also reported to colonize through sialic acid-containing receptors and sialoglycoprotein respectively. *Pseudomonas aeruginosa*, a major pathogen in cystic fibrosis, was shown to adhere to respiratory tissue using sialic acid-containing receptors on cell surfaces or in mucins (59).

Using the Biacore assay to evaluate the interaction between selected probiotics and sulfate residues, *L. gasseri* OLL2877 showed a significant decrease after both

sulfatase and barium chloride treatment suggesting OLL2877 has a strong adhesion to the sulfate expressed in sHCM. A previous report shows mucus-binding proteins of *L. reuteri* showed significant decrease in adherence of human intestinal mucin after sialidase or sulfatase treatment (46). The outer membrane protein of *H. pylori* was reported to bind strongly to sulfo-Lewis a, sulfogalactose and sulfo-GlcNAc in salivary mucin (44); and *P. aeruginosa* also showed strong adherence to salivary mucin containing sulfated glycopeptides (59, 60). The sulfate content of colonic and salivary mucin are similar among the digestive tracts (30). According to these studies and my results, highly sulfated mucin may be a binding site for some microorganisms, although acidomucin has been proposed to protect the mucin from bacterial digestion. Some reports indicated that additional sulfated and/or sialylated polysaccharides suppressed the colonization of *H. pylori in vitro* and *in vivo* (61, 62).

Although the adhesion of OLL2785 strain and *B. bifidum* MCC1092 decreased after blockage of sulfate groups, it increased after sulfatase digestion. From the specific binding position of sulfate groups (Fig. 2-1), OLL2785 strain and *B. bifidum* MCC1092 may have some adherence properties to sulfate. However, they may have more binding abilities to galactose or GlcNAc exposed after sulfatase digestion than to the sulfate residue. Previously, some lactobacilli were reported that strongly bound to blood group antigens expressed in intestinal mucosa, which implied the terminal sugar residues of colonic mucin may serve as binding sites for probiotics (43, 63).

Here, a new screening method was constructed using the Biacore assay to search for high-adhesive probiotics to sialic acid and sulfate residues of human colonic mucin. Three probiotics were found that recognized sialic acid, sulfate, and/or terminal oligosaccharide residues of colonic mucin showing a variation in adhesion

strategy among microbes. Next, the adhesive properties of bacterial cell surface proteins from three probiotics to the sialic acid and sulfate residues were analyzed.

CHAPTER 3

Analysis of surface proteins from probiotics with adhesion properties to acidic residues using the Biacore assay

Introduction

In chapter 2, a new screening method was developed using Biacore adhesive assay to observe the binding of bacteria to the acidic residues of human colonic mucin (HCM). First, some procedures were added in purification of HCM to further study the adhesion of mucin oligosaccharides and terminal residues. The removal of acidic residues and blockage of sulfate were performed by enzymic and chemical treatment, respectively. Finally, three bacterial strains isolated from human feces with strong adhesion to acidic residues were selected. However, the adhesion molecules and adhesive properties of the strains with specific affinity to acidic residues were still unknown.

The adhesive abilities of microflora to GI tract are proved to associate with their cell surface protein (64). In the previous studies using the Biacore adhesion assay, some *Lactobacillus* species isolated from the human intestine were found that adhered to the human blood group A and B antigens of HCM. The cell surface protein, e.g. the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (43) and ATP-binding cassette (ABC) transporter (55) expressed on the surface of bacteria, were demonstrated to participate in the adhesion. There are some methods used for extraction of bacterial surface proteins that researcher have used are: (1) chaotropic agents like LiCl or guanidine, (2) detergents like SDS, (3) enzymes such as trypsin or lysozyme, or (4) buffers used for washing such as PBS (65). After extraction, there are some effective strategies for profiling cell surface protein, e.g. using cell culture, biotinylation of membrane proteins, and analysis by 2-D PAGE (66). In this chapter, the PBS wash fractions and GHCl-extracted fractions of the selected probiotic strains were tested for adhesion to sHCM and the bionylated probes containing acidic residues by Biacore

adhesion assay, which is a label-free technique that allows to detect and monitor the interaction of bacterial surface protein and target ligands in real time. Since a relationship between acidic mucin and microflora was proved, the results in this chapter may be helpful to understand the adhesion strategies of bowel bacteria.

Materials and Methods

1. Preparation of bacterial cells and cell surface proteins

Lactobacillus strain OLL2785, *L. gasseri* OLL2877 and *B. bifidum* MCC1092 were provided from the culture collection of Meiji Co., Ltd. and Morinaga Milk Industry Co., Ltd. as mentioned before. The bacterial cells of the three strains were shown to bind to acidic residues in sHCM (67). The lactobacilli and bifidobacteria strains were propagated twice before the experiments at 37 °C for 16 h with a 2% (v/v) and 10% (v/v) inoculum, respectively. After incubation, bacterial cells were collected from the culture medium using centrifugation (8500 × *g*, 4 °C, 10 min) and washed three times with PBS (pH 7.4). The PBS wash fraction was collected and retained. After removing the media components, the bacterial cells were lyophilized as untreated cells. To prepare cell surface proteins, the bacterial cells were incubated in 2 M GHCl at 37 °C for 2 h. After centrifugation (8500 × *g*, 4 °C, 30 min), the pellet (GHCl-treated bacterial cells) and the supernatant (GHCl-extracted fraction) were dialyzed against distilled water at 4 °C for 48 h and then lyophilized. The PBS wash fraction was also dialyzed and lyophilized.

Before using the Biacore assay, the bacterial cells and cell surface proteins were suspended in HBS-EP buffer (GE Healthcare Bio-Sciences K.K., Tokyo, Japan) as the analyte (3 mg/mL) in the Biacore assay.

2. Identification of *Lactobacillus* strain OLL2785 by 16S rRNA gene sequence

Lactobacillus strain OLL2785 was grown in MRS broth (Difco Laboratories); and propagated once at 37 °C for 16 h with a 1% (v/v) inoculum. After that, OLL2785 `strain was cultured in MRS agar (1.5 % (w/v) agar in MRS broth) at 37 °C for 84 h.

Three bacterial colonies on the agar were selected for direct polymerase chain reaction (PCR). The universal primers 35F and 1492R were used (68). Amplification was performed in a 20 μL reaction mixture containing 10 μL TAKARA Ex Taq (Takara Shuzo), 200 μL 10× Ex Taq buffer, 160 μL dNTP mixture (2.5 mM each), each promer (10 pmol), and bacterial cells. The samples were amplified using a C1000™ Thermal Cycles (Bio-Rad, CA, USA) and the following: 95 °C for 3 min; 30 cycles of 95 °C for 30 s, 50 °C for 30s, and 72 °C for 90 s; and finally 72 °C for 10 min (Table 3-1). After electrophoresis and purification with ExoSAP-IT (GE Healthcare), approximately 500 base pair (bp) of the 5' end of the 16S rRNA gene containing hypervariable regions V1 to V3 was sequenced using the 35F primer, a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA) and an ABI PRISM 3130x1 Genetic Analyzer (Applied Biosystems). Sequences were compared with similar sequences to the reference organisms using EzTaxon server 2.1 (http://147.47.212.35:8080/) or a BLAST search (http://blast.ncbi.nlm.nih.gov/).

3. The growth curve of OLL2785, OLL2877, and MCC1092 strains

To determine the growth curve, lactobacilli and bifidobacteria were propagated in the conditions mentioned before, and were grown in 5 mL broth with 2% (v/v) and 10% (v/v) inoculum, respectively. Growth curve and bacterial concentrations were determined by measuring optical density (OD) at 560 nm each 120 min.

4. The ligands using in Biacore assay

All Biacore experiments were performed using a Biacore 1000 (GE Healthcare Bio-Sciences K.K.) at 25 °C in HBS-EP buffer. sHCM and biotinylated probes were used as ligands immobilized on CM5 and SA sensor chips, respectively.

4-1. Soluble human colonic mucin (sHCM)

Purification and immobilization of sHCM was performed as described in chapter 2 (67). Briefly, crude mucus was scraped from the normal intestinal tissues of colorectal cancer patients. After removing lipids by chloroform: methanol (2:1, v/v) and diethyl ether, the lipid-free mucus was digested with Proteinase K (TaKaRa Biotechnology), and purified by gel filtration chromatography with a Toyopearl HW-65F column (90 × 2.6 cm; Tosoh). Fractions containing high concentrations of sugars and peptides were collected as the ligand for the Biacore analysis. The immobilization of sHCM on a CM5 sensor chip (GE Healthcare Bio-Sciences K.K.) was an amine coupling reaction following the manufacturer's instructions. sHCM was dissolved at 10 mg/mL in 10 mM sodium acetate buffer (pH 4.0) and immobilized using the reaction between NHS-esters and radicals of primary amino groups in sHCM. A blank flow cell on CM5 chip activated by an EDC/NHS mixture and deactivated by ethanolamine hydrochloride was used as control flow cell. This study was approved by the ethics committee of Tohoku University Graduate School of Medicine.

4-2. Biotinylated probes

The following biotinylated probes were used as ligands in this study: α-Neu5Ac-polyacrylamide (PAA)-biotin (Neu5Ac-PB), β-D-Galactose-PAA-biotin (Gal-PB), β-D-Gal-3-sulfate-PAA-biotin (Gal-3-S-PB), β-GlcNAc-PAA-biotin (GlcNAc-PB), and β-D-GlcNAc-6-sulfate-PAA-biotin (GlcNAc-6-S-PB) (GlycoTech, MD, USA) (Fig. 3-1). The polymeric probe was immobilized using biotin-avidin binding reaction to the sensor chip SA. The SA chip was first cleaned with three 1-min injections of 10 μl of a solution of 1M NaCl in 50 mM NaOH before the immobilization procedure.

When the sensorgram reached a stable baseline, the biotinylated probe, diluted in HBS-EP buffer to 40 μg/mL, was injected for 10 min using a flow rate of 10 μL/min. After that, a 1-min injection of 10 μl of a solution of 1M NaCl in 50 mM NaOH was repeated to clear unbound probes. The sensor chip was equilibrated in HBS-EP buffer.

5. The adhesion assay using Biacore

Quantifying adhesion assay using the Biacore 1000 is based on the principle of surface plasmon resonance (53). After washing and lyophilization, bacterial cells and cell surface proteins as analytes were suspended at a concentration of 3 mg/mL in HBS-EP buffer. After injecting bacterial cells or cell surface proteins at a flow rate of 3 μL/min for 5 min, the sensor chip was washed with HBS-EP buffer to remove unbound analyte; and regenerated eluting with 1 M GHCl solution at a flow rate of 3 μL/min for 2 min. The resonance units (RU) were measured for 200 s after the cessation of sample addition. A response of 1 RU represents about 1 pg/mm^2 protein adhering with increased concentration of analyte bound to the sensor chip surface. The overlaid sensorgrams were created using BIAevaluation software pachage (version 3.1).

6. Statistical analysis

All experiments were performed in triplicate and reported as the mean ± SD. For adhesion test of cell surface proteins using Biacore assay, statistical analysis of the data was performed using the Student's *t*-test. For each statistic, significant differences were inferred whenever the *p*-value was <0.05.

Table 3-1. Primers used for sequence of 16S rRNA genes and PCR conditions

Primer	Sequence (5'-3')
35F	CCTGGCTCAGGATGAACG
1492R	GGTTACCTTGTTACGACTT

Component of pre-mixture	Volume (μL)
TaKaRa Ex Taq™ HS polymerase (5 units/μL)	0.1
10× *Ex Taq* buffer	2.0
dNTP mixture	1.6
10 μM 35F primer	0.2
10 μM 1492R primer	0.2
Sterilized distilled water	up to 20.0

PCR cycle		
95 °C	180 sec	
95 °C	30 sec	× 30 cycles
50 °C	30 sec	
72 °C	90 sec	
72 °C	600 sec	
4 °C	∞	

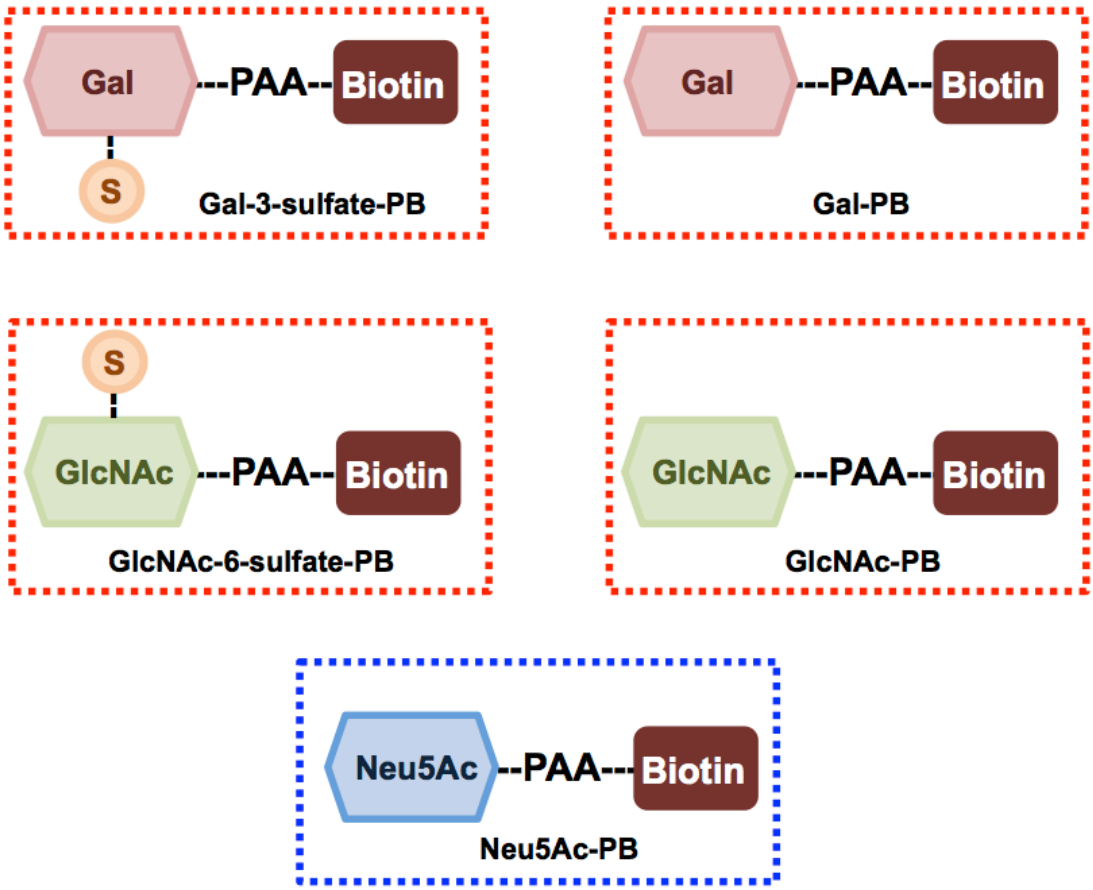


Figure 3-1. The structure of biotinylated probes used in this study. PAA: polyacrylamide, Gal: galactose, GlcNAc: *N*-acetylglucosamine, Neu5Ac: *N*-acetylneuraminic acid, S:sulfate.

Results

1. Identification of *Lactobacillus* strain OLL2785 by 16S rRNA gene sequence

Since the bacterial species of OLL2785 strain was still unknown, the species identification by 16S rRNA gene sequence was performed. After amplified by the universal primers 35F and 1492R, approximately 500 base pair (bp) of the 5' end of the 16S rRNA gene containing hypervariable regions V1 to V3 was sequenced (Fig. 3-2). By comparing with the reference organisms, OLL2785 showed 100% similarity to *L. fornicalis*, 99.281% to *L. psittaci* and *L. jensenii* (Table 3-2). Thus, OLL2785 was identified as *L. fornicalis* in species level identification.

2. The growth curve of *L. fornicalis* OLL2785, *L. gasseri* OLL2877, and *B. bifidum* MCC1092

The growth curve of the three strains was measured after twice propagation. Two lactobacilli strains showed similar growth curve. The log phase started in 2 h and the stationary phase began in 8 to 10 h in *L. fornicalis* OLL2785. In *L. gasseri* OLL2877, the lag phase was not obvious; and the stationary phase started in 10 h. After 18 h incubation, the bacterial cell concentration of *L. fornicalis* OLL2785 and *L. gasseri* OLL2877, observed by optical microscope, came to 1.5×10^{10} and 4.0×10^{9} cells/mL. The growth curve of *B. bifidum* MCC1092 was different from the two lactobacilli. The lag phase ended in 4 h. The log phase was from 4 to 12 h. Until 20 h, the bacterial cell concentration came to 4.0×10^{9} cells/mL (Fig. 3-3).

3. Analysis of adhesive properties of bacterial surface proteins to acidic residues using the Biacore assay

In chapter 2, a new screening method was developed to measure the binding between bacteria and acidic residues in sHCMs using the Biacore binding assay. Two lactobacilli strains and one bifidobacteria strain were selected with strong adhesion to acidic residues of sHCM. Here, I further confirmed the binding between the cell surface proteins of the two lactobacilli and sHCM. To quantify the binding between the cell surface proteins and the specific structure of acidic residues, the biotinylated probes containing the structure of acidic residues in HCM were used as ligands in Biacore assay in this study.

3-1. The binding between cell surface proteins and sHCM

To confirm whether the adhesin-like components were removed by GHCl extraction, the adhesion value of the GHCl-treated and untreated bacteria were compared in the Biacore adhesion assay. Untreated OLL2877 cells adhered to sHCM at 323.1 ± 58.9 RU, whereas a significant reduction was shown with GHCl-treated cells to sHCM at 1.8 ± 0.5 RU ($p < 0.01$). The unsmooth curve of OLL2877 was due to the aggregation property (Fig. 3-4). The OLL2785 and MCC1092 strains also showed a significant reduction of adhesion value in GHCl-treated cells (data not shown), suggesting the adhesin-like components were extracted by GHCl solution.

Here, the PBS wash fractions and the GHCl-extracted fractions from the strains were tested for adhesion to sHCM and control flow cell. Most of the fractions adhered to sHCM significantly when compared to control ($p < 0.05$). In both OLL2785 and OLL2877 strains, the adhesion values of GHCl-extracted fractions were significantly higher than PBS wash fractions ($p < 0.01$), and the 2M GHCl-extracted fractions showed the highest binding to sHCM among the three kinds of fractions (23.1 ± 5.3

RU and 1972.3 ± 185.5 RU, respectively). However, in MCC1092 strain, the binding of PBS wash fraction was higher than the GHCl fractions (Fig. 3-5).

3-2. The binding between cell surface proteins and acidic residues

After proving the binding ability of cell surface proteins to sHCM, the adhesion between the bacterial cell surface protein and the probes with acidic residues was further determined. Here, SA sensor chip immobilized with the biotinylated probes was used, whereas CM5 sensor chip was used for immobilization of sHCM. The immobilization amounts of Gal-3-S-PB, GlcNAc-6-S-PB and Neu5Ac-PB were 806.5, 785.1, and 164.0 RU, respectively (Table 3-3). From the sensorgrams of immobilization, the association rate of GlcNAc-6-S-PB was higher than Gal-3-S-PB. Neu5Ac-PB was the lowest one (Fig. 3-6).

The adhesion values of cell surface proteins to acidic residues were measured. In both OLL2785 and OLL2877 strains, the adhesion values of 2M GHCl-extracted fractions were significantly higher than PBS wash fractions ($p < 0.05$). The 2M GHCl fractions of OLL2785 and OLL2877 showed highest binding to Gal-3-S (73.0 ± 6.7 RU and 792.1 ± 94.5 RU, respectively). However, in MCC1092, there was no significant difference (Fig. 3-7). Among the three strains as analyte, OLL2877 showed the highest binding to the three acidic probes (Fig. 3-8).

3-3. The binding between cell surface proteins and monosaccharides

The biotinylated probes without acidic residues were used as negative control, which were Gal-PB and GlcNAc-PB. Compared to the corresponding acidic probes, the decrease of immobilized amount in Gal-PB and GlcNAc-PB was due to lower

mass (Table 3-4). The immobilization of Gal-PB, GlcNAc-6-S-PB, and GlcNAc-PB was completed in 50 s, which of Gal-3-S was completed in 400s (Fig. 3-9).

The adhesion values of 2M GHCl-extracted fractions to the biotinylated probes with/without acidic residues were measured. In OLL2785 and OLL2877 strains, the adhesion values of the 2M GHCl fractions to the acidic probes (Gal-3-S-PB and GlcNAc-6-S-PB) were significantly higher than to the probes without acidic residues (Gal-PB and GlcNAc-PB). The adhesion values of 2M GHCl fraction from OLL2877 to Gal-3-S (841.3 ± 12.9 RU) and GlcNAc-6-S (646.5 ± 1.9 RU) were significantly higher when compared to Gal (363.4 ± 0.8 RU) and GlcNAc (429.4 ± 0.9 RU) ($p <$ 0.001). Same as the results of the adherence to sHCM, OLL2877 showed the highest adherence to the acidic probes among the three strains. However, there was no significant difference in MCC1092 (Fig. 3-10). Due to the insolubility of 2M GHCl fraction from MCC1092, the unsmooth curves were observed in the Biacore sensorgrams. The 2M GHCl fraction from OLL2785 showed higher association rate and dissociation rate than which from OLL2877, but the binding values were higher in OLL2877 (Fig. 3-11).

TGCAGTCGAGCGAGCTTGCCTATTGAAATTCTTCGGAATGGACATAGATA

CAAGCTAGCGGCGGATGGGTGAGTAACGCGTGGGTAACCTGCCCTTAA

GTCTGGGATACCATTTGGAAACAGATGCTAATACCGGATAAAAGCTACTT

TCGCATGAAAGAAGTTTAAAAGGCGGCGTAAGCTGTCGCTAAAGGATGG

ACCTGCGATGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGAT

GATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACA

CGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGG

ACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGAT

CGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTT

TATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAG

CCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAA

AGCGAGCGCAGGCGGATTGATAAGTCTGATGTGAAAGCCTTCGGCTCAA

CCGAAGAACTGCATCAGAAACTGTCAATCTTGAGTGCAGAAGAGGAGAG

TGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACC

AGTGGCGAA

Figure 3-2. DNA sequences from the 5' end of the 16S rRNA gene from *Lactobacillus* strain OLL2785 (695 bp).

Table 3-2. 16S rDNA-based identification of OLL2785 strain by Eztaxon. The data are representative of three independent experiments.

Rank	Name/Title	Authors	Strain	Accession	Pairwise Similarity	Diff/Total nt	megaBLAST score	BLASTN score
1	Lactobacillus fornicalis	Dicks et al. 2000	TV1018(T)	Y18654	100.000	0/693	1354	1350
2	Lactobacillus psittaci	Lawson et al. 2001	CCUG 42378(T)	AJ272391	99.281	5/695	1332	1332
3	Lactobacillus jensenii	Gasser et al. 1970	ATCC 25258(T)	AF243176	99.280	5/694	1316	1302
4	Lactobacillus kitasatonis	Mukai et al. 2003	JCM 1039(T)	AB107638	92.507	52/694	1023	999

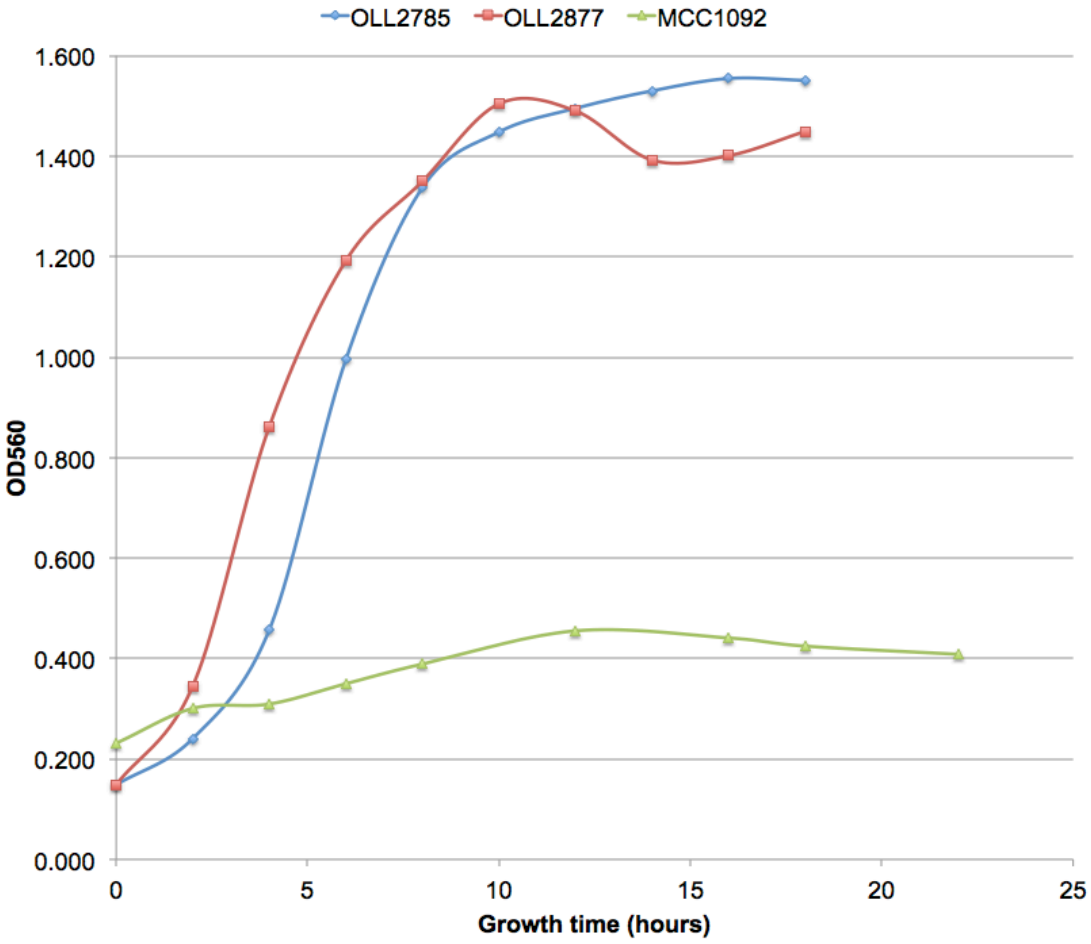


Figure 3-3. Growth curves of *L. fornicalis* OLL2785, *L. gasseri* OLL2877, and *B. bifidum* MCC1092 with 2%, 2%, and 10% (v/v) inoculum, respectively. The measurement of OD were performed each 120 min. The data represent means (n = 3).

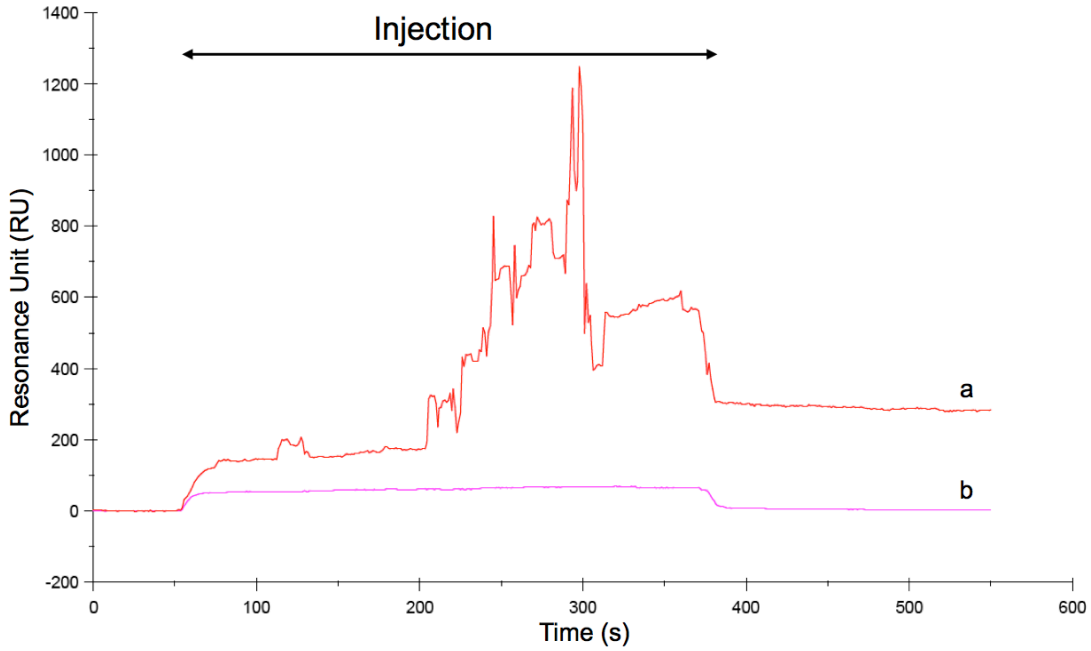


Figure 3-4. Representative overlaid sensorgrams illustrating adhesion assay of untreated (panel a) and GHCl-extracted bacterial cells (panel b) from *L. gasseri* OLL2877 to sHCM.

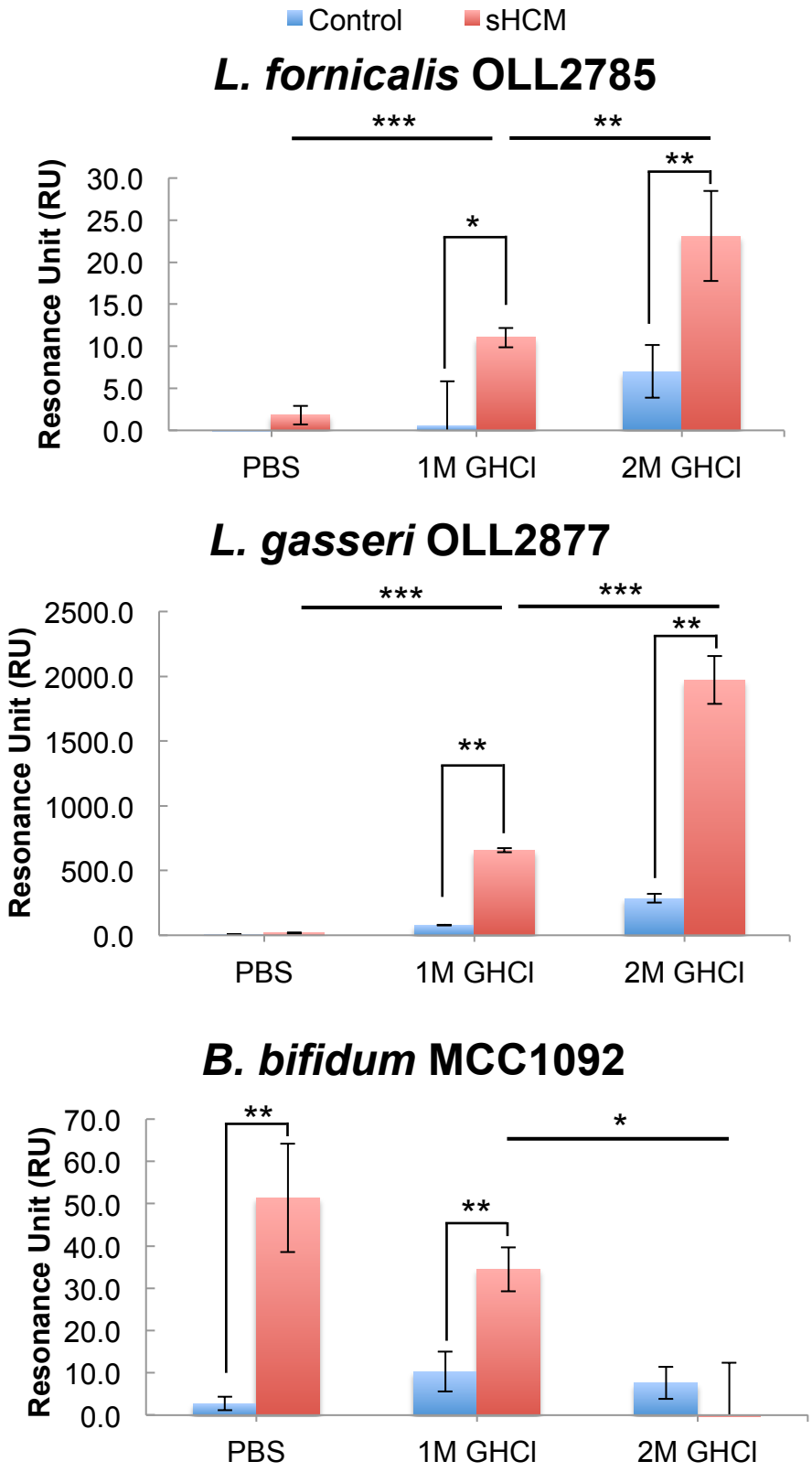


Figure 3-5. Binding abilities of the bacterial cell surface proteins to sHCM using the Biacore assay. 'PBS' and 'GHCl' are PBS wash fraction and the GHCl-treated fraction of each strain, respectively. Columns represent the mean ± SD (n = 3).*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 3-3. Probes used for determining the binding abilities of the surface proteins of lactobacilli and bifidobacteria to the biotinylated probes containing acidic residues, and their immobilized amount to sensor chip SA.

FC1: β-D-Gal-3-sulfate-PAA-biotin	Sulfate probe
FC2: β-D-GlcNAc-6-sulfate-PAA-biotin	Sulfate probe
FC3: α-Neu5Ac-PAA-biotin	Neu5Ac probe
FC4: Blank	

SA sensor chip	FC1	FC2	FC3	FC4
	Gal-3-sulfate	GlcNAc-6-sulfate	Neu5Ac	Blank
Immobilized amount (RU)	806.5	785.1	164.0	0

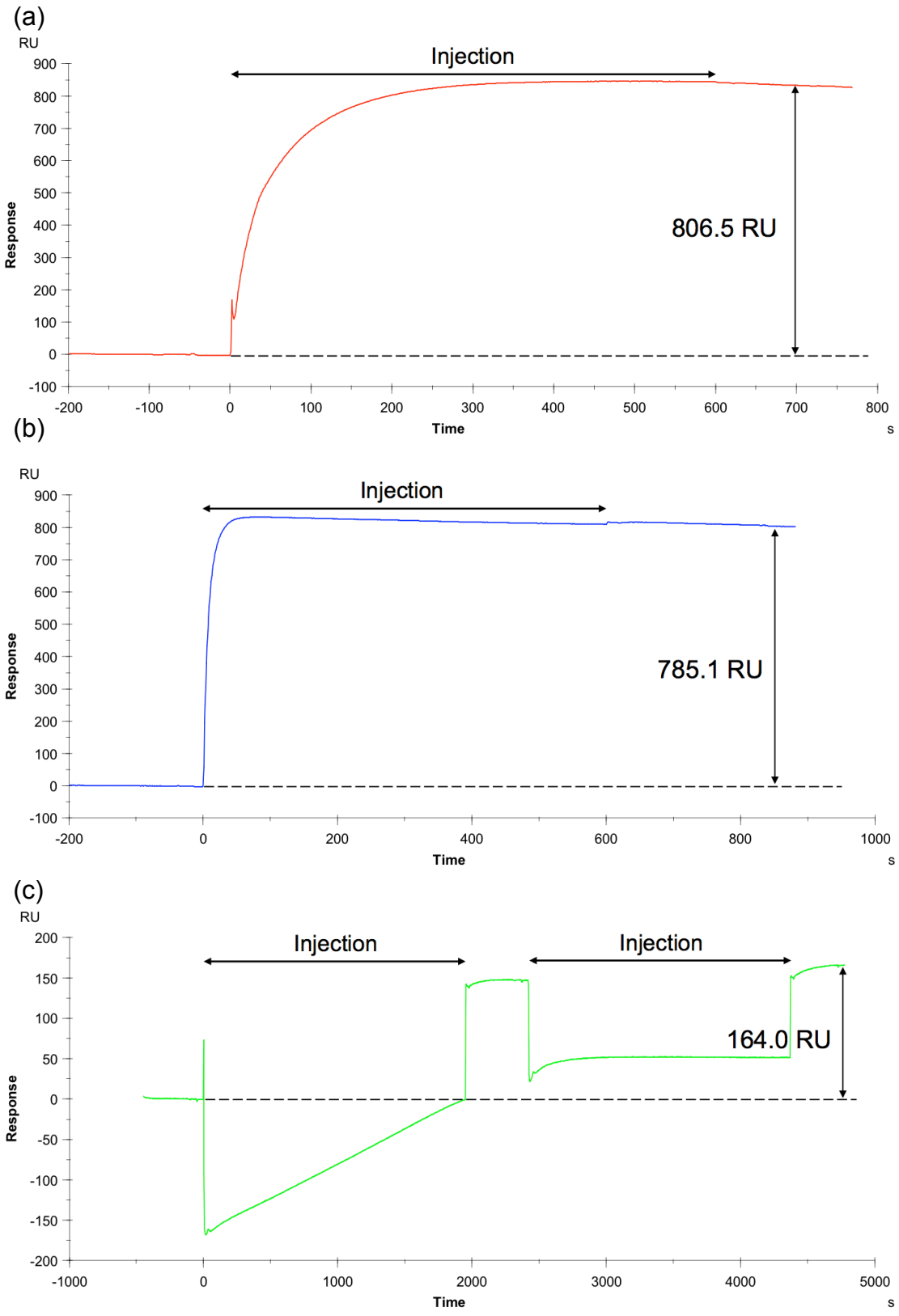


Figure 3-6. Biacore sensorgrams of immobilization of (a) Gal-3-S-PB, (b) GlcNAc-6-S-PB, and (c) Neu5Ac-PB on the sensor chip SA.

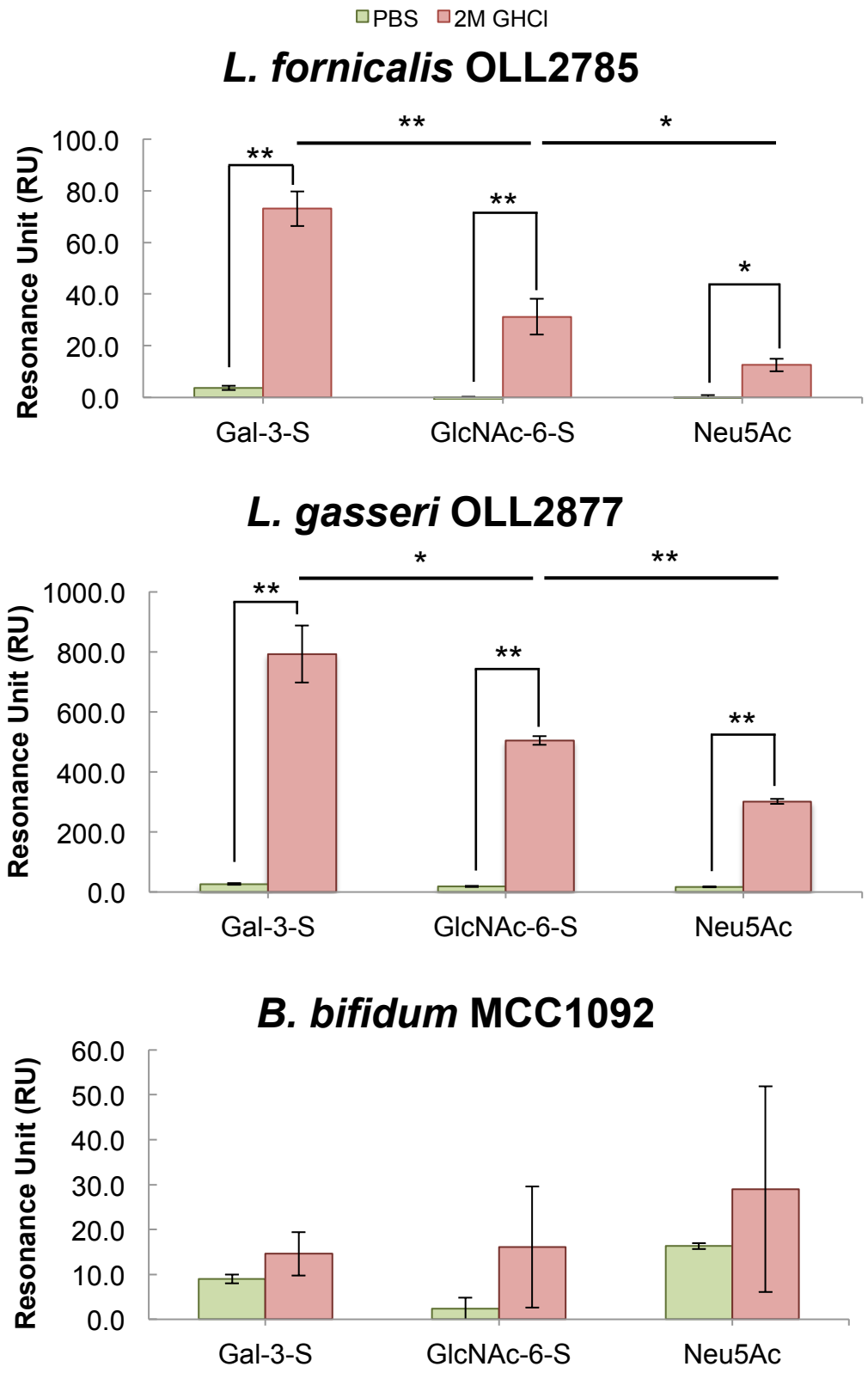


Figure 3-7. Binding abilities of the PBS wash fraction and the 2M GHCl extract fraction of the selected strains to the biotinylated probes containing acidic residues using Biacore analysis. Columns represent the mean ± SD (n = 3). *$p < 0.05$; **$p < 0.01$.

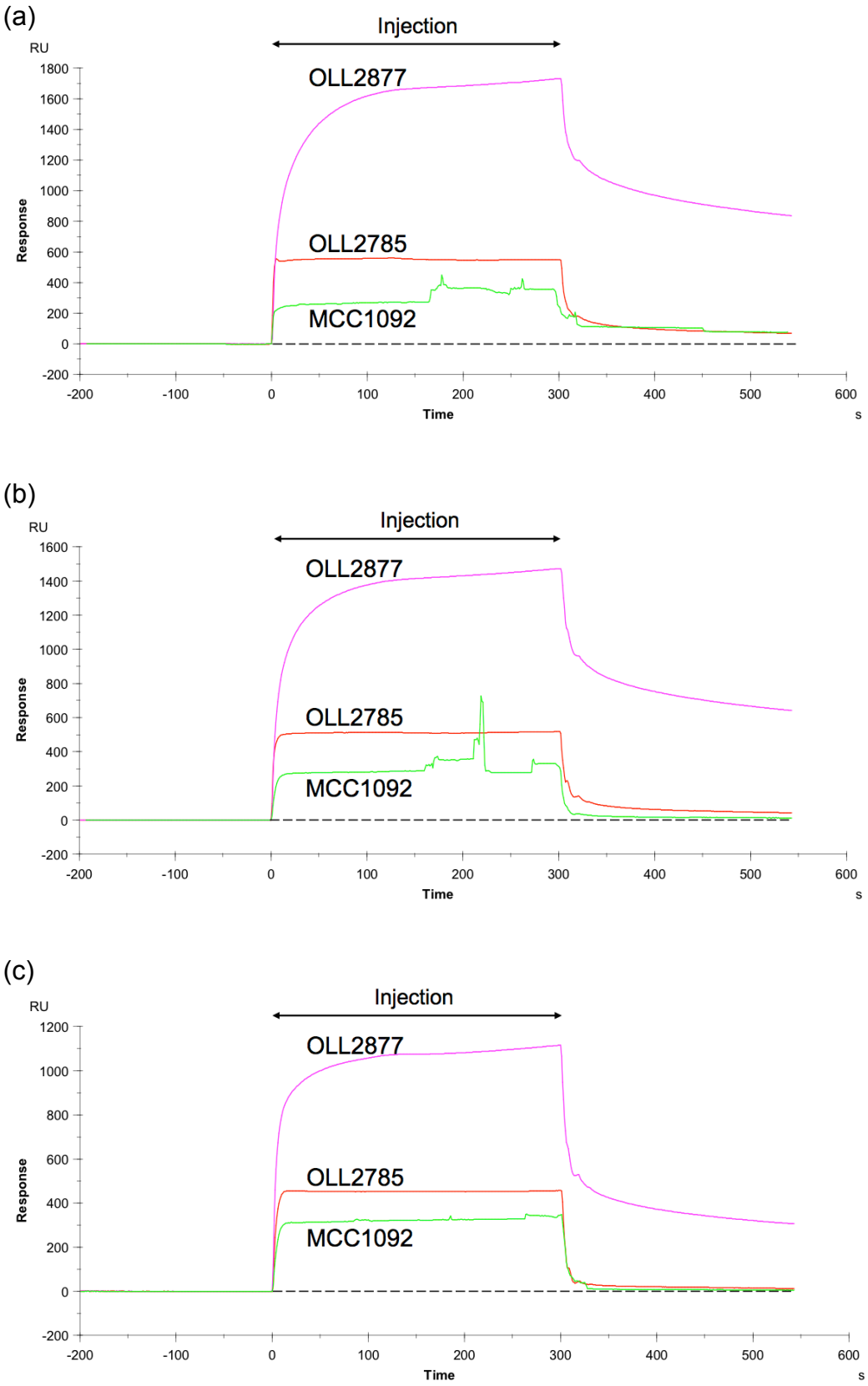


Figure 3-8. Representative overlaid sensorgrams illustrating adhesion assay of 2M GHCl extract fraction of the selected strains to (a) Gal-3-S-PB, (b) GlcNAc-6-S-PB, and (c) Neu5Ac.

Table 3-4. Probes used for determining the binding abilities of the surface proteins of lactobacilli and bifidobacteria to the biotinylated probes containing sulfate residues and the control probes. The immobilized amounts to sensor chip SA also show below.

FC1: β-D-Gal-3-sulfate-PAA-biotin	Gal-3-sulfate and Gal probe
FC2: β-D-Gal-PAA-biotin	
FC3: β-D-GlcNAc-6-sulfate-PAA-biotin	GlcNAc-6-sulfate and GlcNAc probe
FC4: β-D-GlcNAc-PAA-biotin	

SA sensor chip	FC1	FC2	FC3	FC4
	Gal-3-sulfate	Gal	GlcNAc-6-sulfate	GlcNAc
Immobilized amount (RU)	914.8	857.6	986.1	937.2

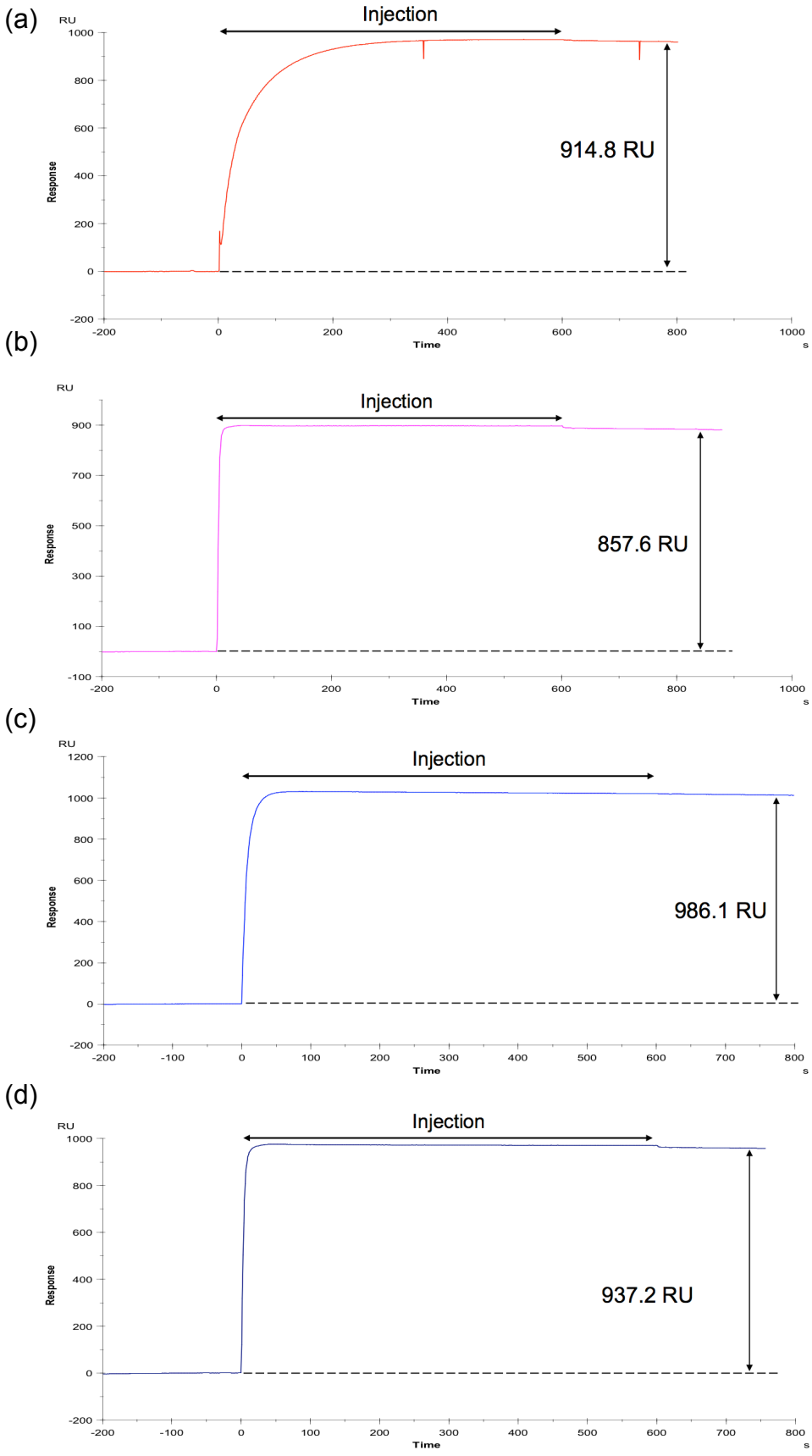


Figure 3-9. Biacore sensorgrams of immobilization of (a) Gal-3-PB, (b) Gal-PB, (c) GlcNAc-6-S-PB, and (d) GlcNAc-PB on the sensor chip SA.

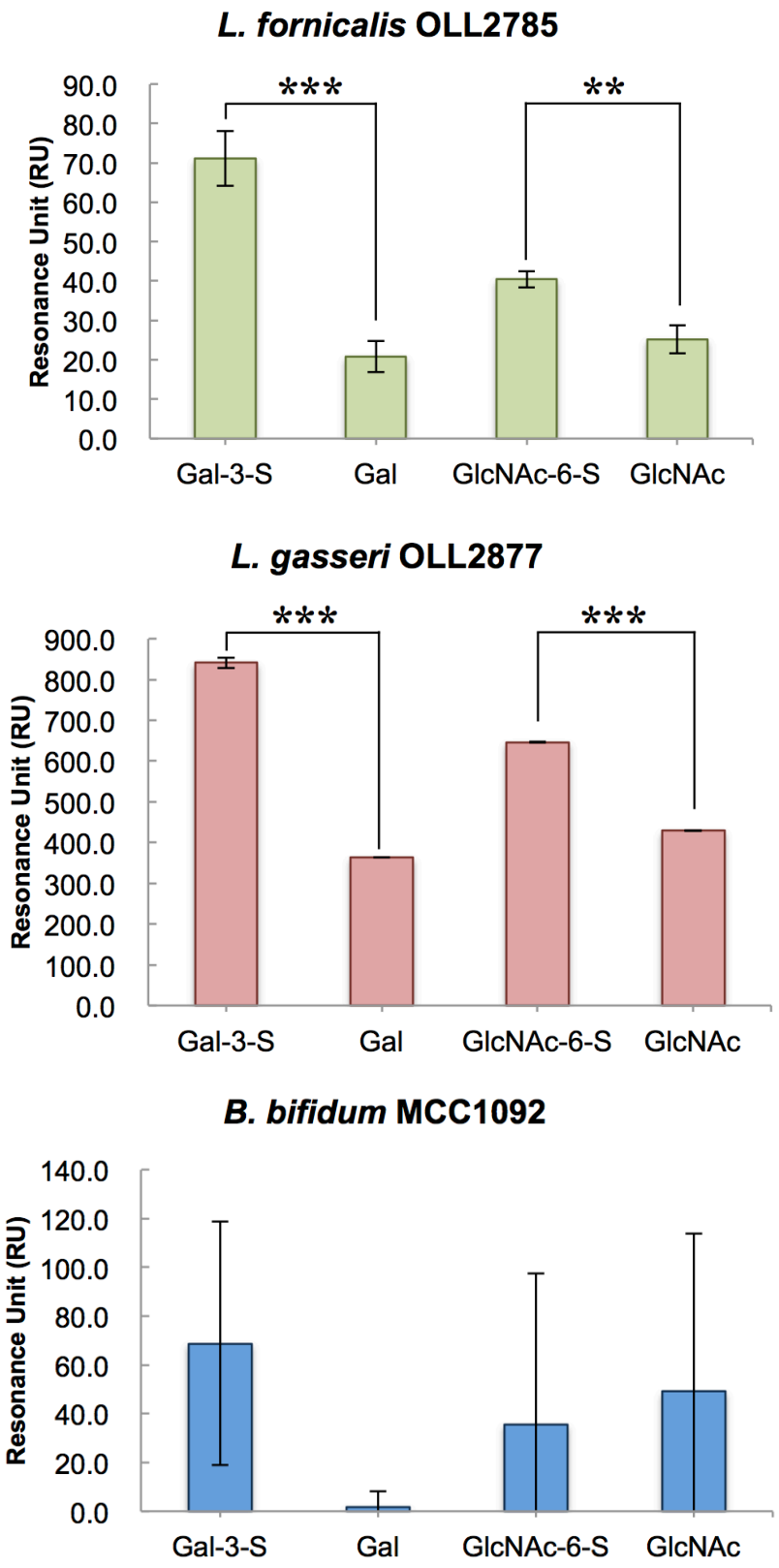


Figure 3-10. Binding abilities of the 2M GHCl extract fractions of the selected strains to the biotinylated probes containing sulfate residue and the control probes using Biacore analysis. Columns represent the mean ± SD (n = 3). **$p < 0.01$; ***$p < 0.001$.

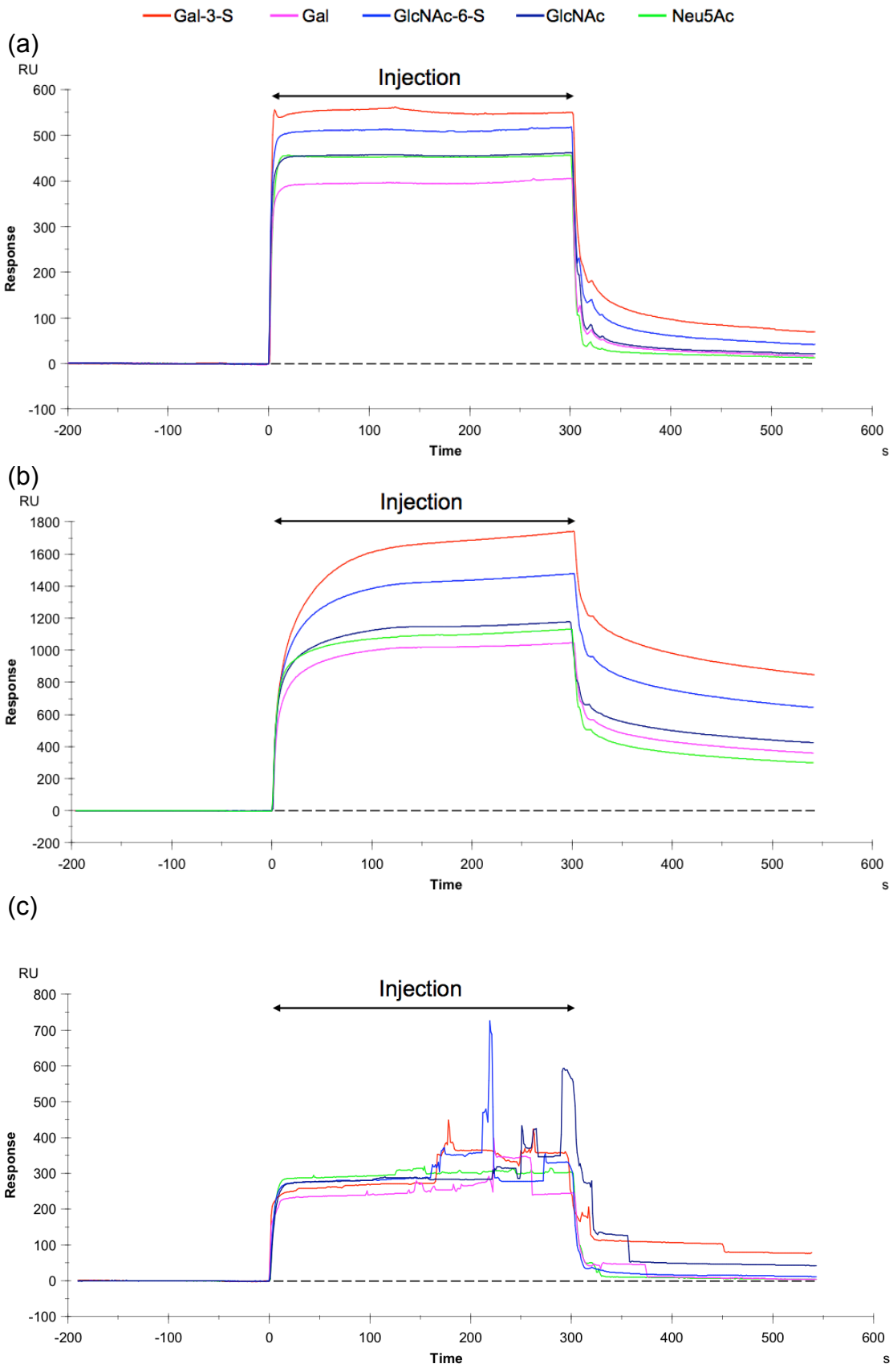


Figure 3-11. Representative overlaid sensorgrams illustrating adhesion assay of 2M GHCl extract fraction of (a) *L. fornicalis* OLL2785, (b) *L. gasseri* OLL2877, and (c) *B. bifidum* MCC1092 to the biotinylated probes.

Discussion

Adhesive characteristics of bacteria vary among strains and species (69). Though specific mechanisms are still unclear, there are some interactions proved to be involved in intestinal mucus adhesion. The most studied one is carbohydrate-protein interaction. The cell surface protein produced by bacteria, such as mucus-binding protein (MUB) by *L. reuteri* (70), pili subunit from *L. rhamnosus* GG (71), and elongation factor Tu (EF-Tu) (72), plays an important role in the adhesion to mucin-bound oligosaccharides. Since Neu5Ac and sulfate residues are predominantly linked to HCM, a new screening method to evaluate the binding of probiotics to acidic residues was constructed in chapter 2. Following the results, three high-adhesive probiotics binding to acidic residues were selected for further studies. In this chapter, to clarify their adhesive properties, I tried to prepare cell surface proteins from the selected probiotics, and measured their binding to the biotinylated probes with/without acidic residues of HCM using the Biacore adhesion assay.

The species level identification of *Lactobacillus* strain OLL2785 and the growth curves of three probiotics were determined at first. OLL2785 was identified as *L. fornicalis* using 16S rRNA gene sequencing. *L. fornicalis* was isolated from the posterior fornix fluid of the human vagina, which is a non-motile, non-sporulating, catalase-negative and oxidase-negative bacterium with optimum temperature on growth between 35 and 37 °C. Also, it is facultatively anaerobic and obligately homofermentative with no gas production from glucose (73). In this study, OLL2785 was isolated from human feces. Results showed the stationary phase of *L. fornicalis* OLL2785 began in 8 to 10 h after 2% inoculation, which was similar to that of *L. gasseri* OLL2877 (10 h) but different from that of *B. bifidum* MCC1092 (12 h). Thus,

the preparation of cell surface protein was performed after 16 h incubation to collect bacterial cells in the stationary phase.

Here, the binding between bacteria surface proteins and sHCM were measured using Biacore. By comparing the binding of untreated and 2M GHCl-treated cells to sHCM, it was determined that the adhesin-like components on cell surface were extracted by 2M GHCl. For OLL2785 and OLL2877, the adhesion values of 2M GHCl-extracted fractions were significantly higher than PBS wash fractions ($p < 0.05$), suggesting the adhesin-like components may exist in 2M GHCl-extracted fractions. For *B. bifidum* MCC1092, the adhesion value of PBS wash fraction was higher than GHCl fraction. It suggests that the adhesin-like components on cell surface of MCC1092 were washed off in the step of three-time washing by PBS. Compared to MCC1092, the adhesion values of PBS wash fraction from OLL2785 and OLL2877 were extremely low (< 20 RU). However, when the binding of surface protein to acidic residues was measured, I found the adhesion value of PBS wash fraction was lower than 2M GHCl-extracted fraction from MCC1092, indicating that the adhesin-like components in PBS wash fraction bound to parts of sHCM besides acidic residues. On the other hand, the 2M GHCl fractions from OLL2785 and OLL2877 showed significantly higher binding than PBS wash fraction in both adhesion assay to sHCM and acidic residues ($p < 0.05$). Thus, the binding to acidic residues contributed to the higher binding of both lactobacilli to sHCM.

Since the binding values of the 2M GHCl fractions from OLL2785 and OLL2877 strains to sulfate probes were significantly higher than those to Neu5Ac probe ($p < 0.05$), the binding to the biotinylated probes with/without sulfate residue was also determined. By comparing the binding to Gal-3-S/GlcNAc-6-S and Gal/GlcNAc, the effect of sulfate residue on adhesion could be clarified. In OLL2785 and OLL2877,

the binding to Gal-3-S was significantly higher than that to GlcNAc-6-S, but the adhesion values to Gal were lower than that to GlcNAc. It suggests that the higher binding to Gal-3-S than GlcNAc-6-S was not contributed by the different monosaccharide liked to sulfate. The whole structure of Gal-3-S may be recognized by adhesin-like components from OLL2785 and OLL2877, because Gal-3-S is the most predominant structure in human colonic acidomucin (29).

Few studies concern the adhesins from probiotics binding to sulfomucin, but some adhesins from pathogens were reported to show specific binding to sulfate residue expressed in mucin. The piliated strain of *Pseudomonas aeruginosa* bound more strongly to highly sulfated salivary mucin from cystic fibrosis than the nonpiliated strain (60), and *P. aeruginosa* flagellin were found to be an adhesin for MUC1 mucin (74). *H. pylori* was reported to adhere to SO_3-3-Gal in salivary mucin (75), and a neutrophil-activating protein expressed on its surface was identified to bind specifically to sulfated oligosaccharides in salivary mucin (44). Furthermore, a report indicated that the binding of *H. pylori* urease to gastric epithelial cells was decreased after the addition of sulfated polysaccharides *in vitro*, indicating the urease had specific binding to sulfate derivatives (76).

Moreover, studies also indicated sialic acid may be a potential binding site for bacteria. *H. pylori* is well known to adhere sialyl-Lewis x antigens on gastric epithelium by sialic acid-binding adhesin (SabA) (57). The K88 fimbrial adhesin expressed on the surface of enterotoxigenic *Escherichia coli* (ETEC) showed binding to sialoglycoproteins from porcine intestine (58). Also, M protein from *Streptococcus pyogenes* (45) and Hsa protein (203 kDa) from *S. gordonii* (77) were also reported to bind to the sialic acid moieties on mucin. Although the adhesion values to Neu5Ac

were lower than sulfate residue in this study, the existence of Neu5Ac-binding adhesin from probiotics is still possible.

In the next chapter, the adhesin-like components were detected on the bacterial cell body *in situ*, and identified using receptor overlay and N-terminal amino acid sequencing analyses.

CHAPTER 4

Detection and identification of adhesin-like components from probiotics with specific affinity to acidic residues in human colonic mucin

Introduction

In chapter 3, the cell surface proteins from *L. gasseri* OLL2877 showed highest adhesion values to sHCM and the biotinylated probes containing acidic residues, at least 10 times higher than that from *L. fornicalis* OLL2785 and *B. bifidum* MCC1092. Among the probes containing different acidic residue, the cell surface proteins from *L. gasseri* OLL2877 and *L. fornicalis* OLL2785 showed highest binding to Gal-3-S, which is the most predominant structure in human colonic acidomucin (29). However, the specific molecular from these surface proteins recognized sulfate residue on mucin oligosaccharides was still unknown.

The classical theories of adhesion of bacteria suggest two to five stages in the process, including passive van der Wall's attractive forces, electrostatic interactions, hydrophobic, steric forces, lipoteichoic acid, and frequently active adhesion through the production of specific structures by the bacteria, such as external appendages covered by lectins and/or extracellular polymers (polysaccharides) (78). An adhesion-promoting protein (29 kDa) is present on the surface of *L. fermentum* 104R and involved in the binding to mucus from the small intestine and stomach of piglets (79). From *L. johnsonii* La1, lipoteichoic acid isolated from bacterial cell wall is responsible for the concentration-dependent inhibition of La1 adhesion to Caco-2 cells (80). Lectin-like protein structures were found on the cell surfaces of *L. animalis* and *L. fermentum* (81), and the aggregation-promoting factor protein located on the cell surface were identified and sequenced from *L. johnsonii* and *L. gasseri* (82), which may be involved in the interactions between the lactobacilli and intestinal epithelium. However, very few studies concentrate on the adhesin-like component involved in the adhesion to acidic residues of intestinal mucin.

Therefore, in this chapter, I tried to detect the adhesin-like component on the bacterial cell body *in situ*, and identified the specific molecules binding to Gal-3-S probe using receptor overlay and N-terminal amino acid analyses.

Materials and Methods

1. Bacterial strains and preparation of cell-surface proteins

L. fornicalis OLL2785, *L. gasseri* OLL2877 and *B. bifidum* MCC1092 were propagated twice before the cultivation in 500 mL broth at 37 °C for 16 h with 2%, 2%, and 10% (v/v) inoculum as mentioned before. After incubation, bacterial cells were collected from the culture medium using centrifugation (8500 × *g*, 4 °C, 10 min) and washed three times with PBS (pH 7.4). To prepare cell surface proteins, the bacterial cells were incubated in 2 M GHCl at 37 °C for 2 h. After centrifugation (8500 × *g*, 4 °C, 30 min), the supernatant (2M GHCl-extracted fraction) were dialyzed against distilled water at 4 °C for 48 h and then lyophilized. The samples were stored at -20 °C until use.

2. In situ detection of Gal-3-S-binding components on bacterial surface

2-1. Preparation of bacterial smear on slide glass

An indirect fluorescent labeling of cell surface components of bacteria cells was preformed using the binding of biotinylated probes and fluorochrome-streptavidin conjugate (83) (Fig. 4-1). After incubation in 30 mL broth, the selected strains were collected from the culture medium using centrifuging 10 min at 3500 × *g*, 4 °C. After removing supernatant, the bacterial cells were washed two times with 10 mL PBS. The pellet was resuspended in PBS to a final cell concentration of 5×10^7 cells/mL using a bacteria counter (AS ONE corporation, Osaka, Japan). To prepare the smears for the test, 10 μL bacterial suspension was transferred to the center of a clean slide (Matsunami Glass Ind., Ltd., Osaka, Japan), and spread out to achieve a thin layer using a loop. After air dry, the slide was submerged in a Coplin jar containing 95%

ethanol (Wako Pure Chemical Industries) for 1 min at room temperature, and excess ethanol was drained off. Finally, the slide was dipped into PBS in a Coplin jar, and heated to 55 °C for 10 min. Slides can be stored up to 1 year at -70 °C at this step.

2-2. Fluorescent staining of bacteria

The Gal-3-S-PB probe was diluted into 50 μg/mL. Smears were covered with 200 μL diluted probe, placed in a petri dish with moistened kimwipes, and incubated at room temperature overnight. The slides were rinsed in 20 mL PBS to remove any debris and incubated in a petri dish filled with 20 mL PBS for 30 min at room temperature. Then, the smears were covered with 200 μL Alexa Fluor 488 streptavidin conjugate (Invitrogen, CA, USA) diluted into 10 μg/mL, and incubated for 1 h at room temperature in dark. From this step, the slides were protected from light carefully. The slides were rinsed and incubated in PBS again for 30 min at room temperature. After that, the smears were covered with 200 μL 4', 6-diamidino-2-phenylindole (DAPI, Dojindo Molecular Technologies, Kumamoto, Japan) diluted into 1 μg/mL, and incubated for 5-10 min at room temperature. After rinsed and incubated in PBS again for 30 min at room temperature, the slides were rinsed by distilled water and allowed to air dry. To preserve the smears, 20 μL mounting medium (Thermo) and an 18 mm × 18 mm coverslip (Matsunami Glass Ind., Ltd.) were added to the slides. After the mounting medium was completely dry, the slides were sealed by applying clear fingernail polish around the sides of the coverslip. The slides were stored in the dark at room temperature. Negative control was similarly operated by replacing Gal-3-S-PB probe with PBS.

2-3. Observation and quantification of fluorescent-labeled bacteria

The slides were observed using a laser scanning microscope LSM700 (Carl Zeiss Microscopy, Cambridge, UK). Imaging data were analyzed using ImageJ software (NIH freeware, available at http://rsb.info.nih.gov/ij/download.html) to quantify fluorescence from all experiments. The area stained by DAPI (blue) and Alexa Fluor 488 (green) in images were selected by using the "Threshold" function, and counted by using "Analyze Particles" function. Blue fluorescence readings (DAPI) were used as a control to count the bacteria number in the microscope field of view (160 μm × 160 μm). The readings of the green fluorescent images (Alexa Fluor 488) were used to quantify the cell surface components binding to Gal-3-S-PB probe by measuring "Total Area" in the field of view. Using the equation below, the intensity of Gal-3-S-binding components per bacterial cell was calculated.

$$y\ (\mu m^2/\text{cell}) = \frac{\text{Total Area}}{\text{Particle counts}}$$

3. SDS-PAGE analysis

Cell surface proteins of each bacterial strain were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis according to the method of Larmmli (84). The polyacrylamide slab gel was prepared using a discontinuous buffer system with a 4.5% stacking gel and a 12.5% separation gel (10 × 12 cm). Molecular marker proteins and samples were dissolved in SDS buffer (62.5 mM Tris, 2% SDS, β-mercaptoethanol, 0.001% bromophenol blue and 10% glycerol, pH 6.8), following by heating at 99 °C for 10 min. SDS-PAGE was performed using an X Cell SureLock™ Electrophoresis Cell (Invitrogen) at a constant voltage of 125 V with a Power PAC 3000 (Bio-Rad) in running buffer containing 0.025 M Tris, 0.2 M glycine and 0.1% SDS. Proteins were visualized with Coomassie Brilliant Blue

(CBB) (Rapid CBB; Kanto Chemical Co., Inc., Tokyo, Japan). SeeBlue® Plus2 pre-stained standard (Invitrogen) was used as molecular weight marker.

4. Receptor overlay analysis

A modified receptor overlay (RO) protein binding assay adapted from (85) was used (Fig. 4-2). Cell surface proteins were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Blots were blocked in SuperBlock Blocking buffer (Thermo Scientific, CA, USA) for 30 min, washed with Tris-buffered saline buffer (20 mM Tris, 150 mM NaCl, pH 8.2) containing 0.05% Tween 20 (TBS-T), and incubated overnight with the biotinylated probes containing acidic residues (Neu5Ac-PB, Gal-3-S-PB, and GlcNAc-6-S-PB) at 5 μg/mL in TBS-T buffer at room temperature. A negative control was similarly operated that the blots were incubated with TBS-T buffer overnight. The blots were then washed and further incubated for 30 min with ExtraAvidin-Alkaline Phosphatase (Sigma-Aldrich) at a 1:30,000 dilution. The blots were again washed, and probe-binding proteins were visualized with ECL reagents (Invitrogen).

5. N-terminal amino acid sequence analysis

After SDS-PAGE analysis and transferred to a PVDF membrane, the proteins were stained with CBB, excised from PVDF membrane, and destained with 100% methanol. The N-terminal amino acid sequence was determined using an ABI Procise 491HT protein sequencer (Applied Biosystems), and BLASTP search was performed (http://blast.ncbi.nlm.nih.gov/).

6. Statistical analysis

All experiments were performed in triplicate and reported as the mean ± SD. For the fluorescent detection of Gal-3-S-binding components on bacterial surface, statistical analysis of the data was performed using the Student's *t*-test. For each statistic, significant differences were inferred whenever the *p*-value was <0.05.

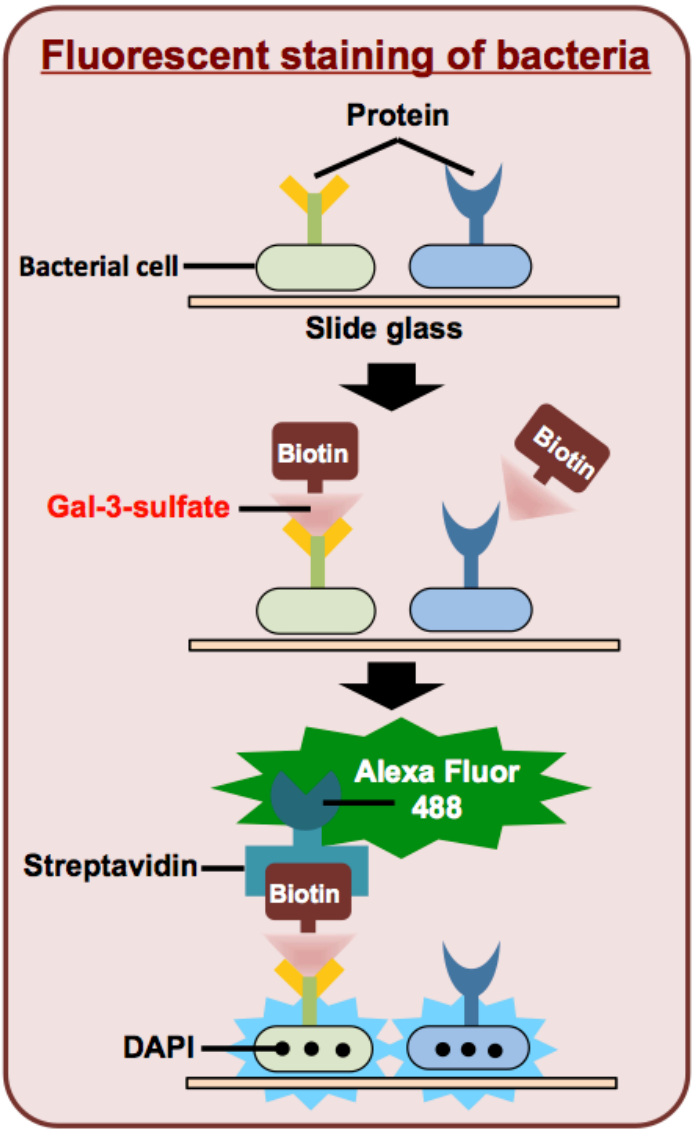


Figure 4-1. The illustration of fluorescent staining of bacteria using Gal-3-S-PB probe.

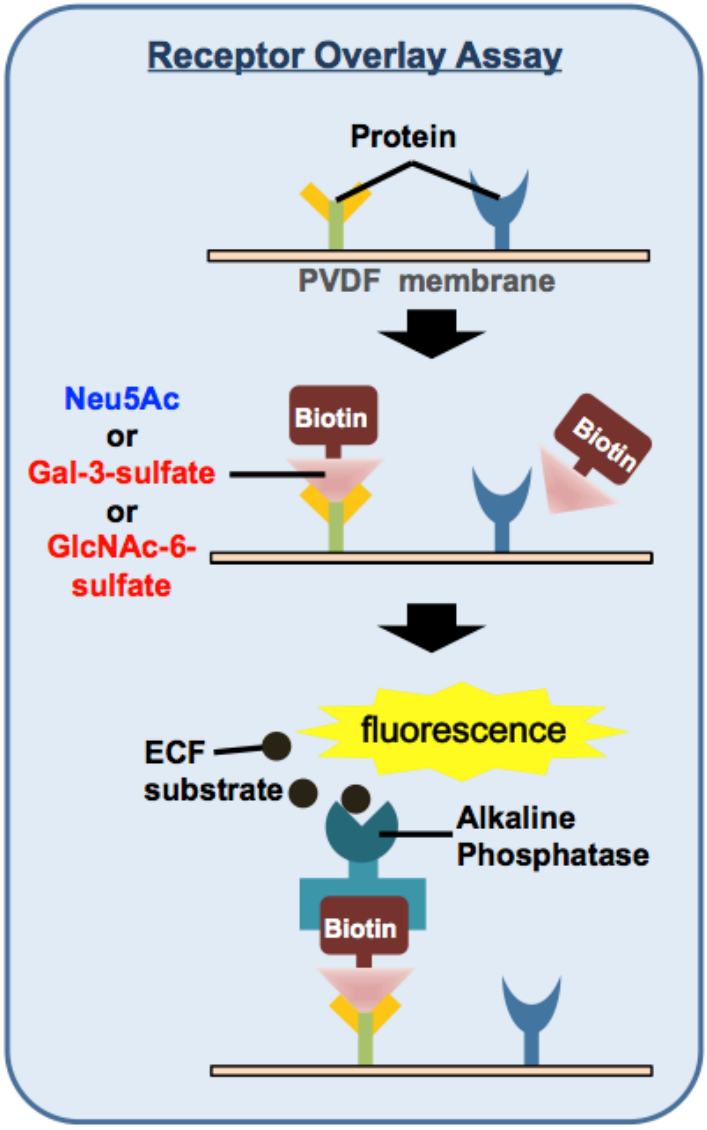


Figure 4-2. The illustration of receptor overlay analysis using biotinylated probes with acidic residues.

Results

1. In situ Detection of bacterial surface proteins with specific affinity to Gal-3-S using indirect fluorescent labeling

The smears were fixed on slide glasses by 95% ethanol. Using optical microscope, the microscopic characteristics of lactobacilli and bifidobacteria were observed (Fig. 4-3). Since the Alexa Fluor 488 signals were very few in negative control, unspecific binding from streptavidin was excluded (Fig. 4-4). Using laser scanning microscope, the Gal-3-S-binding components were observed by fluorescence signals (Fig. 4-5). Under higher magnification, some bacterial cells of *L. fornicalis* OLL2785 were stained with Gal-3-S probe, suggesting the Gal-3-S-binding components were contained on the surface of bacterial cell body. For *B. bifidum* MCC1092, some short cells were stained. However, under flurorescence microscope, most of the Gal-3-S-binding signals were around bacterial cell body of *L. gasseri* OLL2877 (Fig. 4-6).

By analyzing the fluorescence images, the Alexa Fluor 488 signals were quantified and normalized by counting the number of particles stained by DAPI. The fluorescent area per cell of *L. gasseri* OLL2877 (0.62 ± 0.20 μm^2/cell) was significantly higher than which of *L. fornicalis* OLL2785 (0.26 ± 0.15 μm^2/cell) and *B. bifidum* MCC1092 (0.18 ± 0.19 μm^2/cell) ($p < 0.01$). There was no significant difference between *L. fornicalis* OLL2785 and *B. bifidum* MCC1092 (Fig. 4-7).

2. Detection of cell surface components from the probiotics with specific binding activity to acidic residues using receptor overlay analysis

Using receptor overlay analysis with different biotinylated probes, the molecule with specific binding to different probes could be detected. Multiple bands were

observed in the 2M GHCl-extracted fractions of the strains after Coomassie Brilliant Blue staining. The molecular weight of the components from *L. fornicalis* OLL2785 detected by acidic probes was mostly under 36 kDa. There were five bands detected by sulfate probes (Fig. 4-8). The components from *L. gasseri* OLL2877 detected showed a wide-range molecular weight from 64 to 8 kDa. There were eight bands judged as positive detection by sulfate probes (Fig. 4-9). Finally, there were seven molecules from *B. bifidum* MCC1092 judged as positive detection by sulfate probes, ranging from 36 to 8 kDa. The 64-kDa protein from *B. bifidum* MCC1092 was excluded from positive detection because of its strong binding to streptavidin (Fig. 4-10).

In the same bacterial strain, similar patterns were generally observed when the bands detected by Gal-3-S or GlcNAc-6-S probes. Also, the bands detected by Neu5Ac probe were different from sulfate probes, and fewer than that detected by sulfate probes.

3. Identification of the new surface proteins from *L. gasseri* OLL2877 with specific binding activity to sulfate residue

Since the surface proteins from OLL2877 showed the highest adherence to sHCM and acidic residues, the specific components from the 2M GHCl fraction of *L. gasseri* OLL2877 binding to acidic residues were further identified. Compared to the negative control that incubated with TBS-T buffer overnight, I focused on the five bands that showed specific binding to sulfate probes, which were at 64, 63, 34, 29, and 27 kDa (Fig. 4-9).

The molecules with specific binding to sulfate probes were electrically transferred to a PVDF membrane, and the N-terminal amino acids were sequenced. Ten amino

acids residues from the N-terminal end of each molecule were identified (Fig. 4-11, representatively). Using the comparison of the sequences to the database, the 64-kDa and 63-kDa proteins showed 78% and 60% identity with identical protein pyruvate kinase, respectively. The 34-kDa and 29-kDa proteins showed 100% and 90% identity with 50S ribosomal protein L2 and L3, respectively. Finally, the 27-kDa protein showed 88% identity with 30S ribosomal protein S3 (Table 4-1).

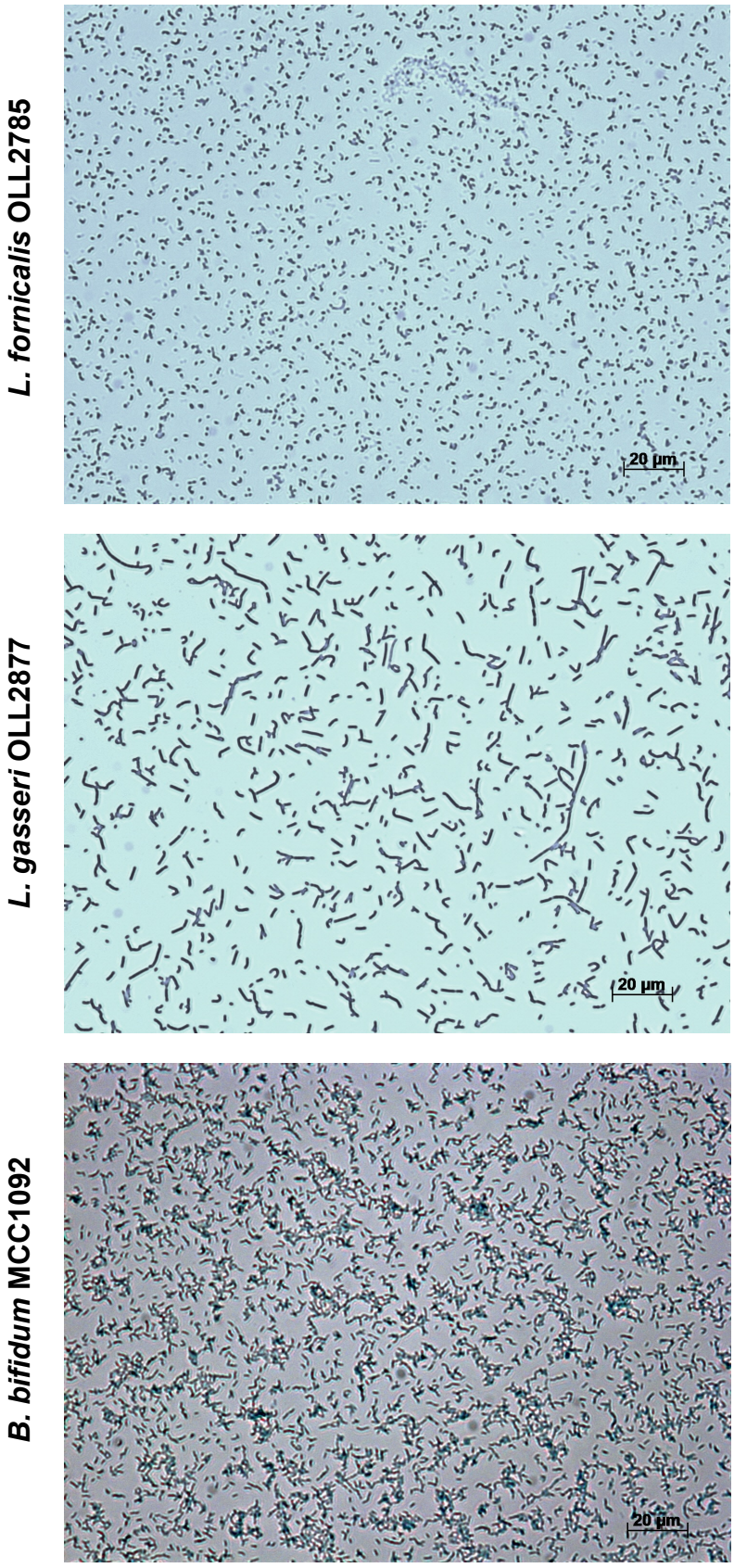


Figure 4-3. Representative images of *L. fornicalis* OLL2785, *L. gasseri* OLL2877, and *B. bifidum* MCC1092 without staining observed by optical microscope.

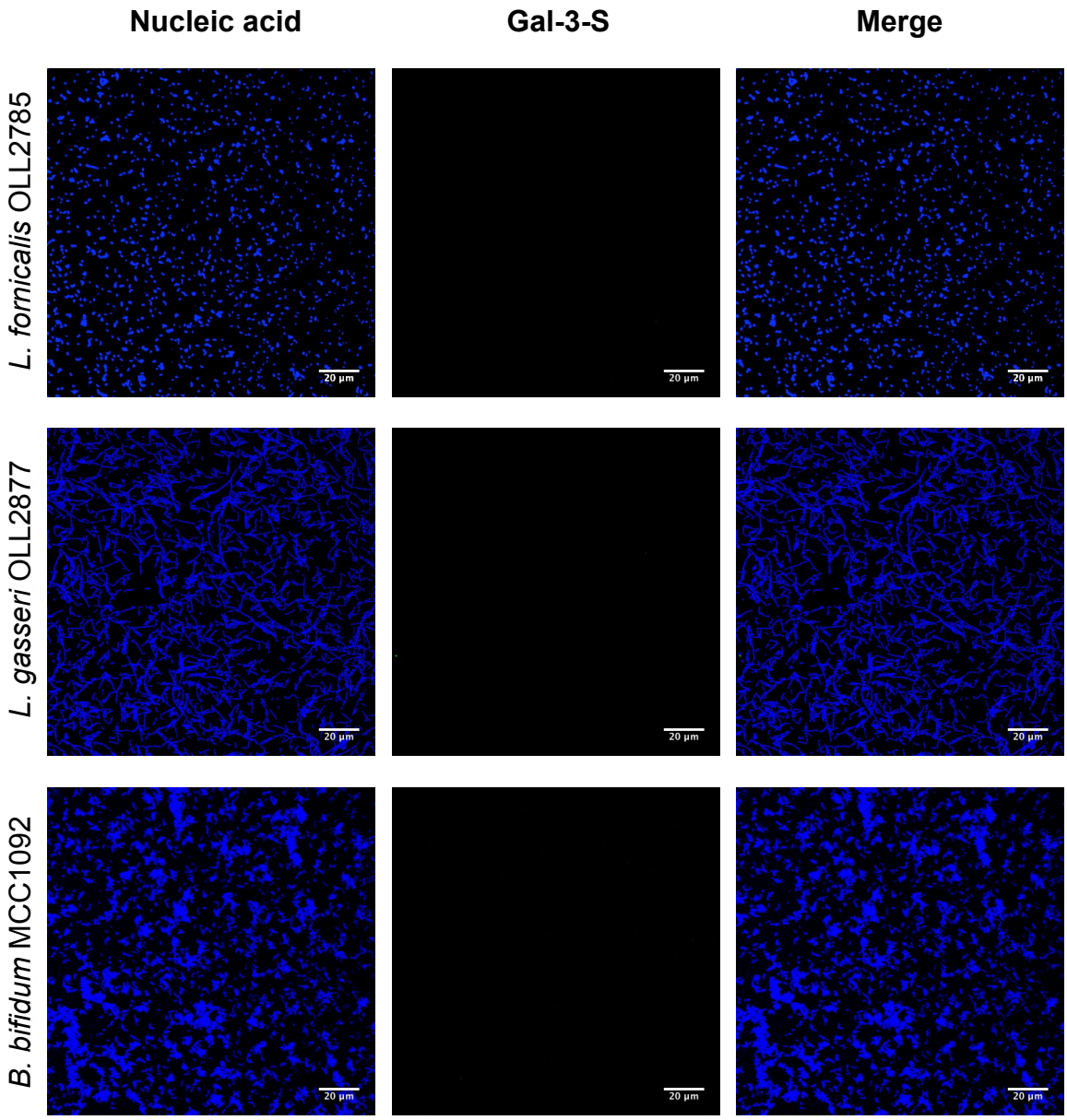


Figure 4-4. Representative images of *L. fornicalis* OLL2785, *L. gasseri* OLL2877, and *B. bifidum* MCC1092 stained by Alexa Fluor 488 streptavidin conjugate (green) and DAPI (blue) under a 400X view as negative control.

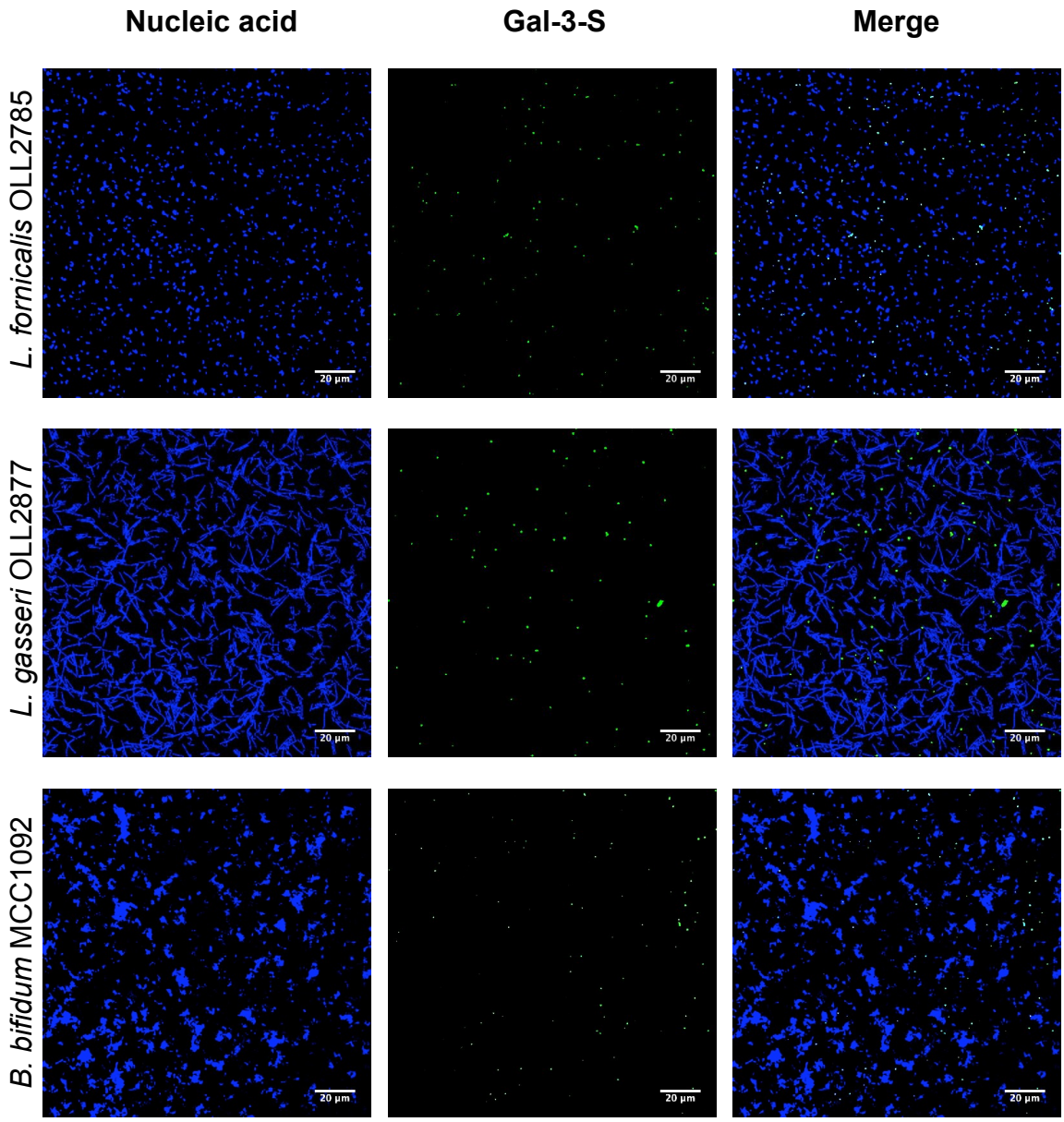


Figure 4-5. Representative images of *L. fornicalis* OLL2785, *L. gasseri* OLL2877, and *B. bifidum* MCC1092 stained by indirect fluorescent labeling (using Gal-3-S-PAA-biotin probe and Alexa Fluor 488 streptavidin conjugate, green) and DAPI (blue) under a 400X view.

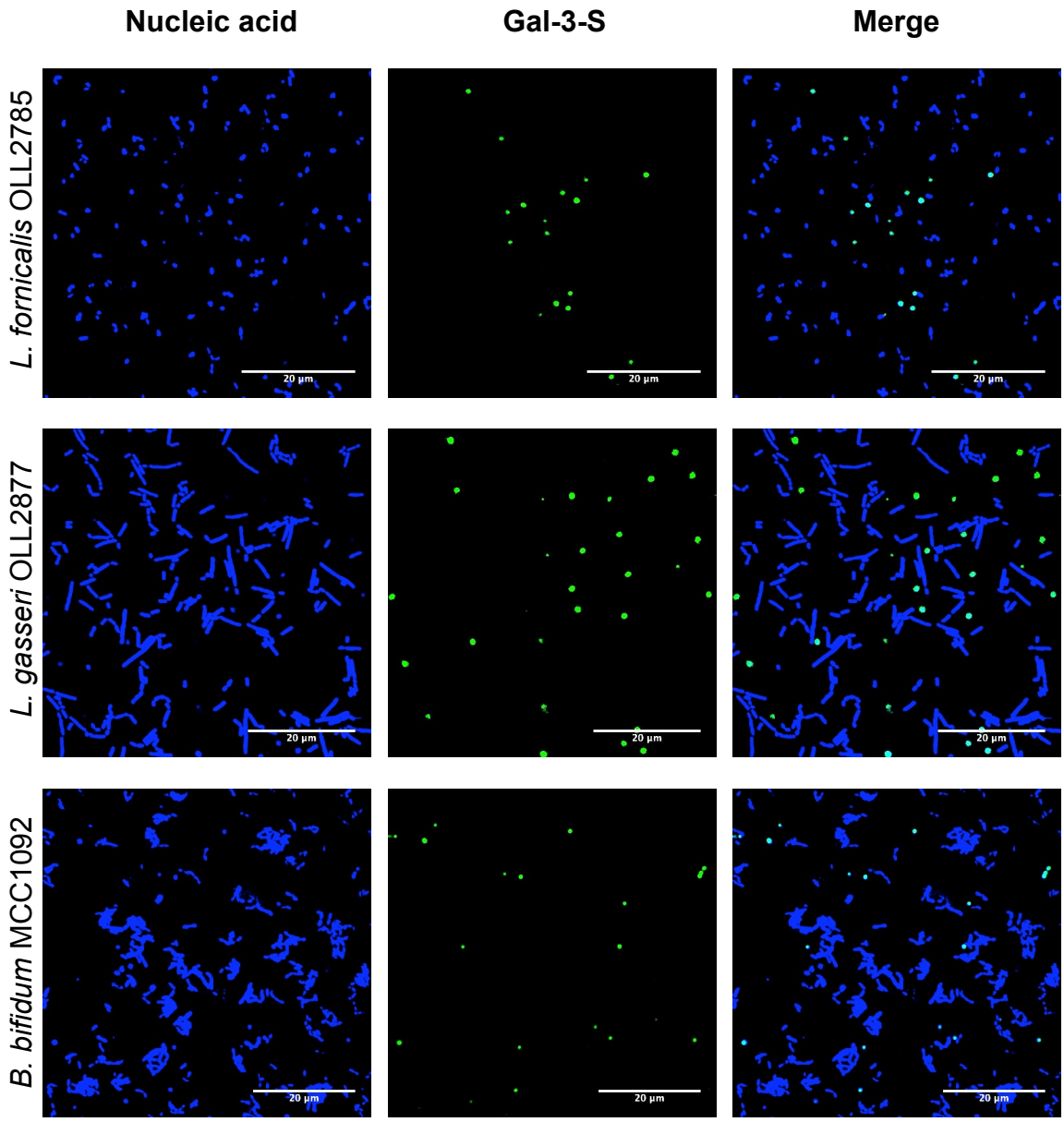


Figure 4-6. Representative images of *L. fornicalis* OLL2785, *L. gasseri* OLL2877, and *B. bifidum* MCC1092 stained by indirect fluorescent labeling (using Gal-3-S-PAA-biotin probe and Alexa Fluor 488 streptavidin conjugate, green) and DAPI (blue) under high magnification.

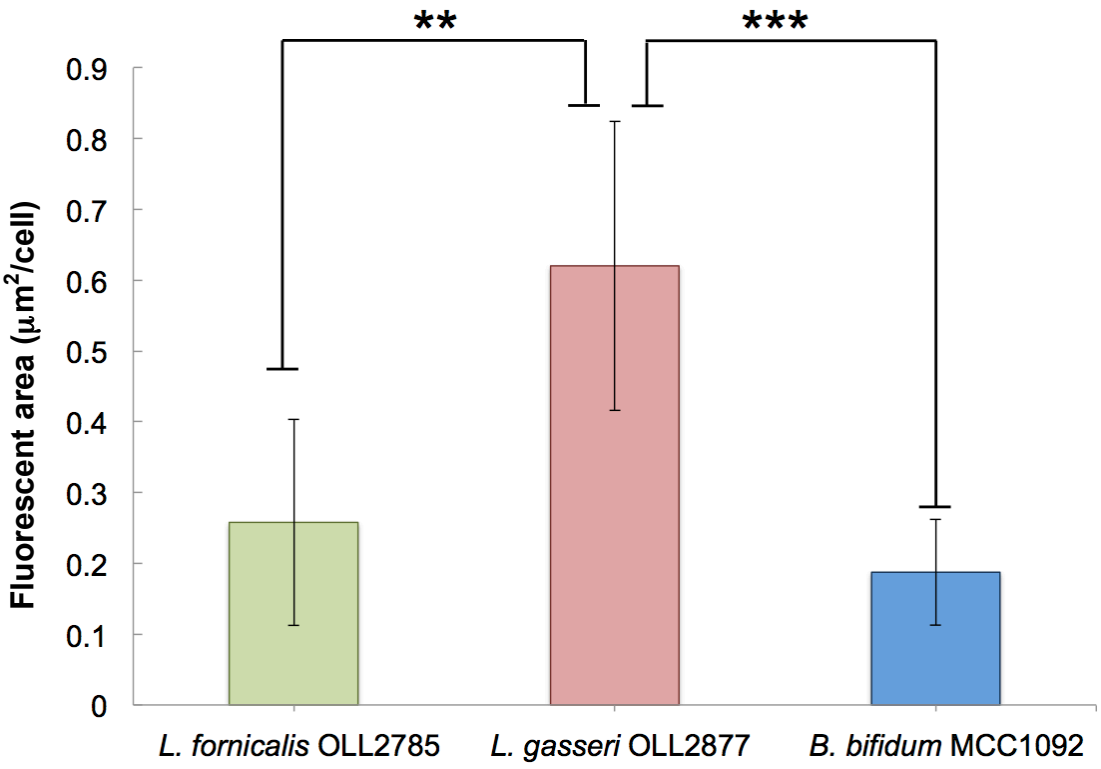


Figure 4-7. Quantification of Alexa Fluor 488 fluorescence of the selected strains with a concentration of 5 × 10^7 cells/mL in 160 μm × 160 μm fields of view. Columns represent means ± SD (n = 5). **$p < 0.01$; ***$p < 0.001$.

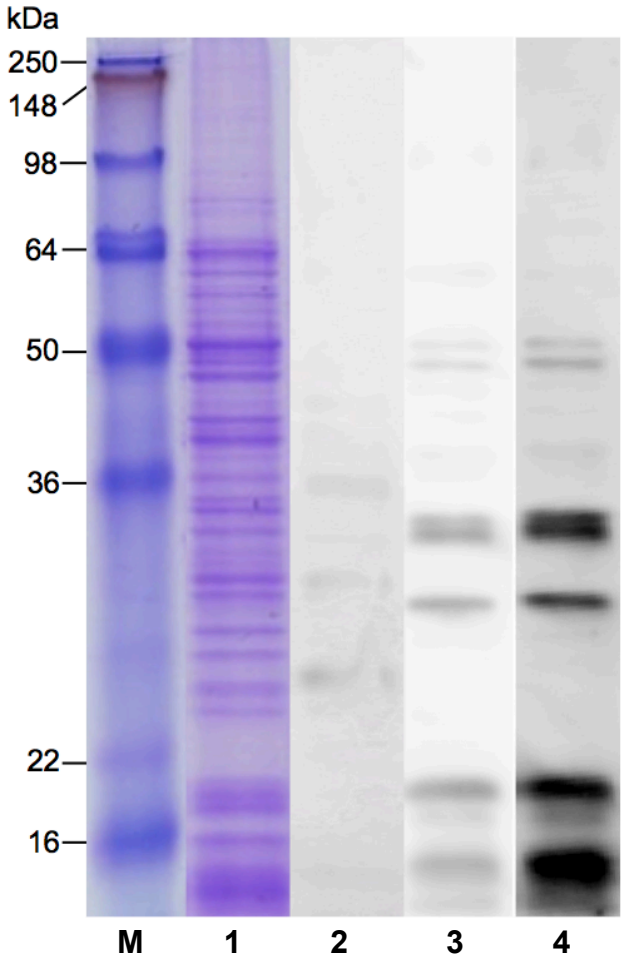


Figure 4-8. Detection of acidic residue-binding molecules in the 2M GHCl fraction of *L. fornicalis* OLL2785 (M: pre-stained molecular weight marker, lane 1: Coomassie Brilliant Blue-stained bands from the 2M GHCl fraction of *L. fornicalis* OLL2785, lane 2: receptor overlay assay using Neu5Ac probe, lane 3: receptor overlay assay using Gal-3-S probe, lane 4: receptor overlay assay using GlcNAc-6-S probe). The data are representative of three independent experiments.

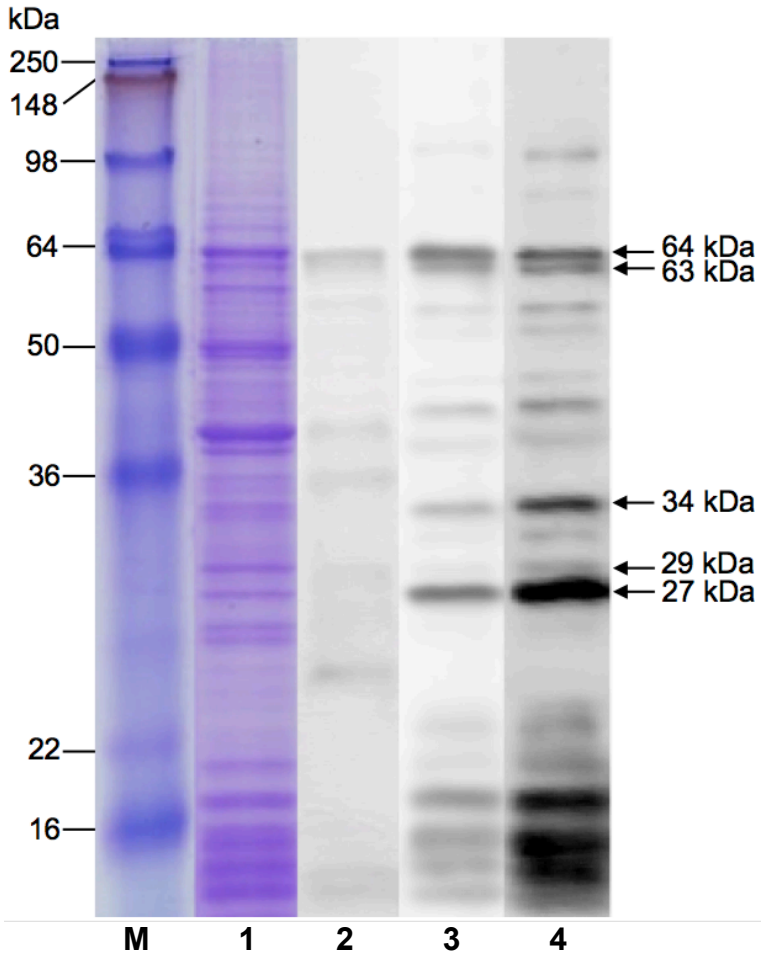


Figure 4-9. Detection of acidic residue-binding molecules in the 2M GHCl fraction of *L. gasseri* OLL2877 (M: pre-stained molecular weight marker, lane 1: Coomassie Brilliant Blue-stained bands from the 2M GHCl fraction of *L. gasseri* OLL2877, lane 2: receptor overlay assay using Neu5Ac probe, lane 3: receptor overlay assay using Gal-3-S probe, lane 4: receptor overlay assay using GlcNAc-6-S probe). The data are representative of three independent experiments.

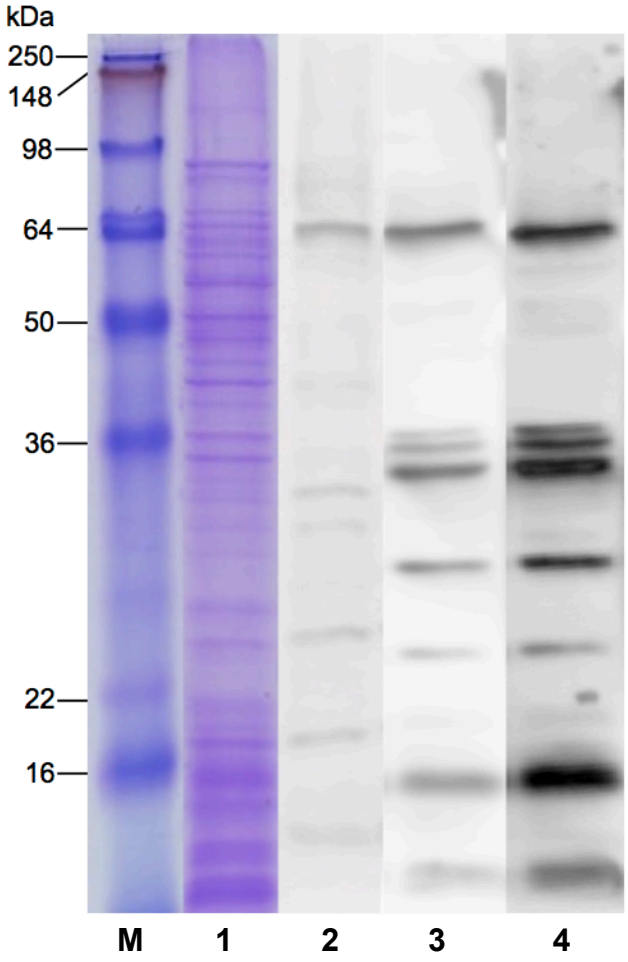


Figure 4-10. Detection of acidic residue-binding molecules in the 2M GHCl fraction of *B. bifidum* MCC1092 (M: pre-stained molecular weight marker, lane 1: Coomassie Brilliant Blue-stained bands from the 2M GHCl fraction of *B. bifidum* MCC1092, lane 2: receptor overlay assay using Neu5Ac probe, lane 3: receptor overlay assay using Gal-3-S probe, lane 4: receptor overlay assay using GlcNAc-6-S probe). The data are representative of three independent experiments.

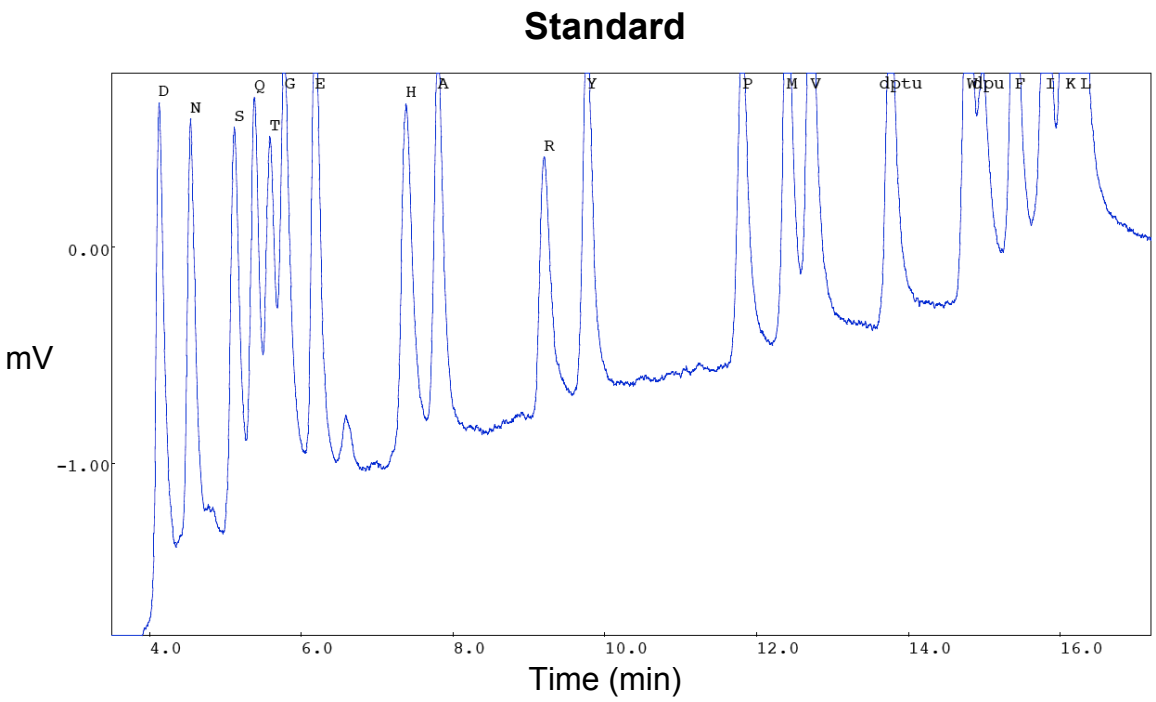


Figure 4-11. HPLC chromatogram of standard amino acid

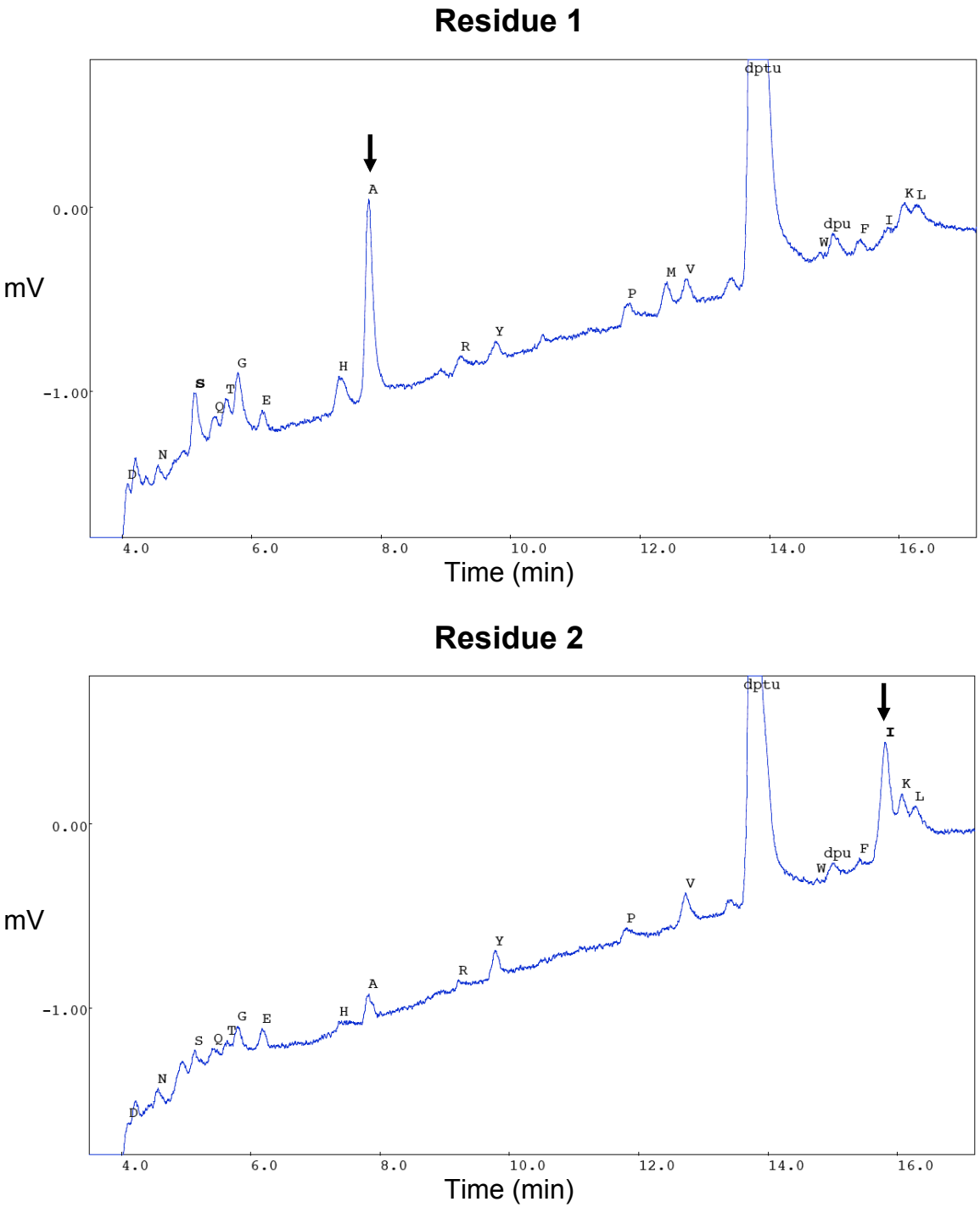


Figure 4-11. HPLC chromatogram of N-terminal amino acid residue from the 34-kDa protein in the 2M GHCl-extracted fraction of *L. gasseri* OLL2877 (residues 1 and 2).

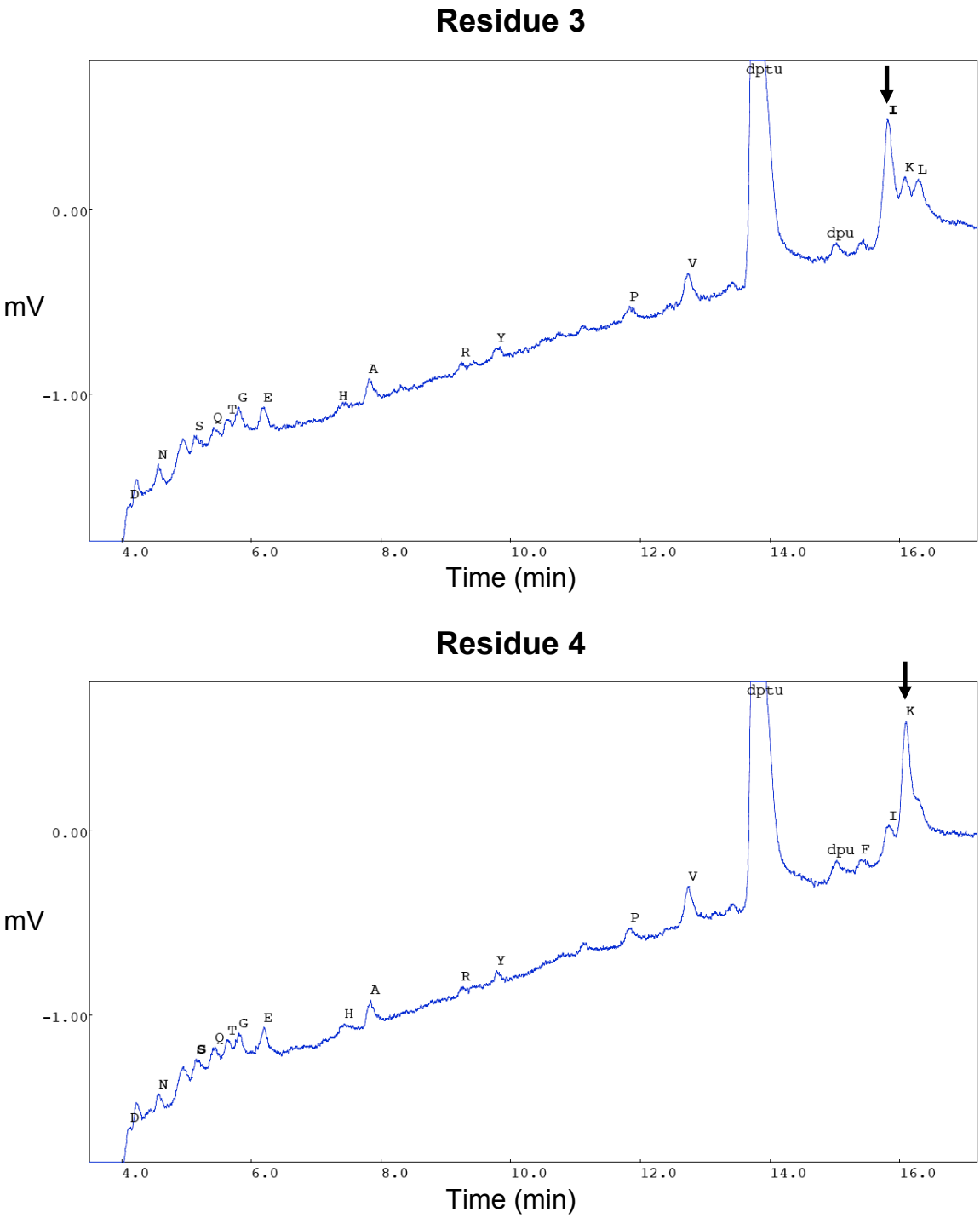


Figure 4-11. HPLC chromatogram of N-terminal amino acid residue from the 34-kDa protein in the 2M GHCl-extracted fraction of *L. gasseri* OLL2877 (residues 3 and 4).

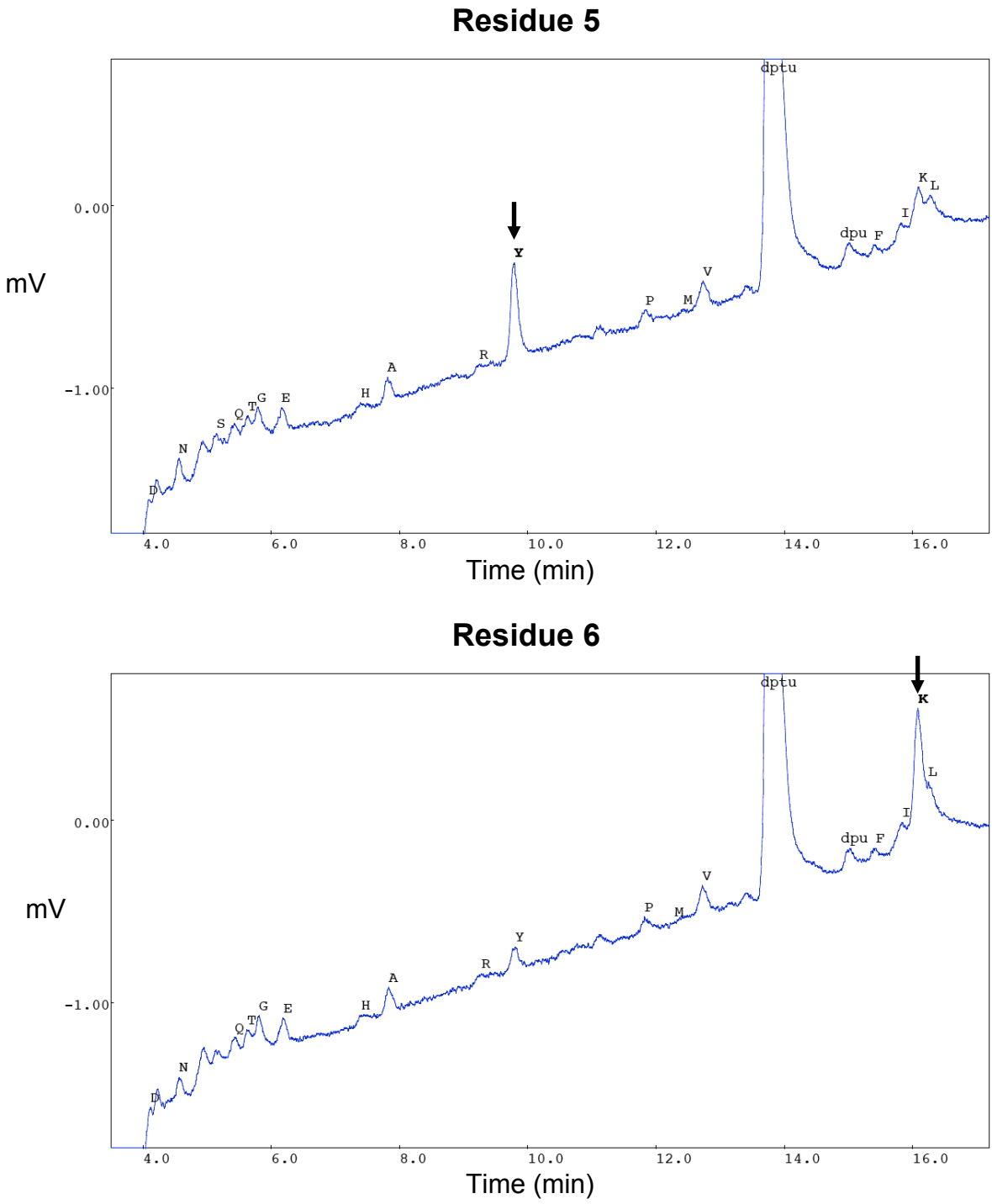
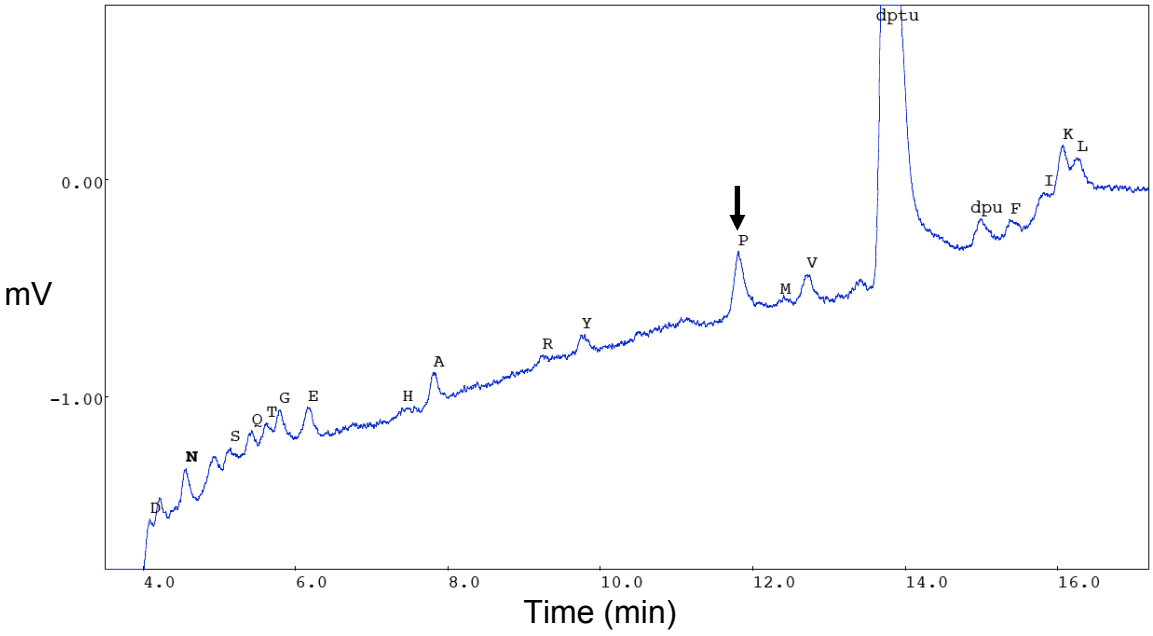


Figure 4-11. HPLC chromatogram of N-terminal amino acid residue from the 34-kDa protein in the 2M GHCl-extracted fraction of *L. gasseri* OLL2877 (residues 5 and 6).

Residue 7



Residue 8

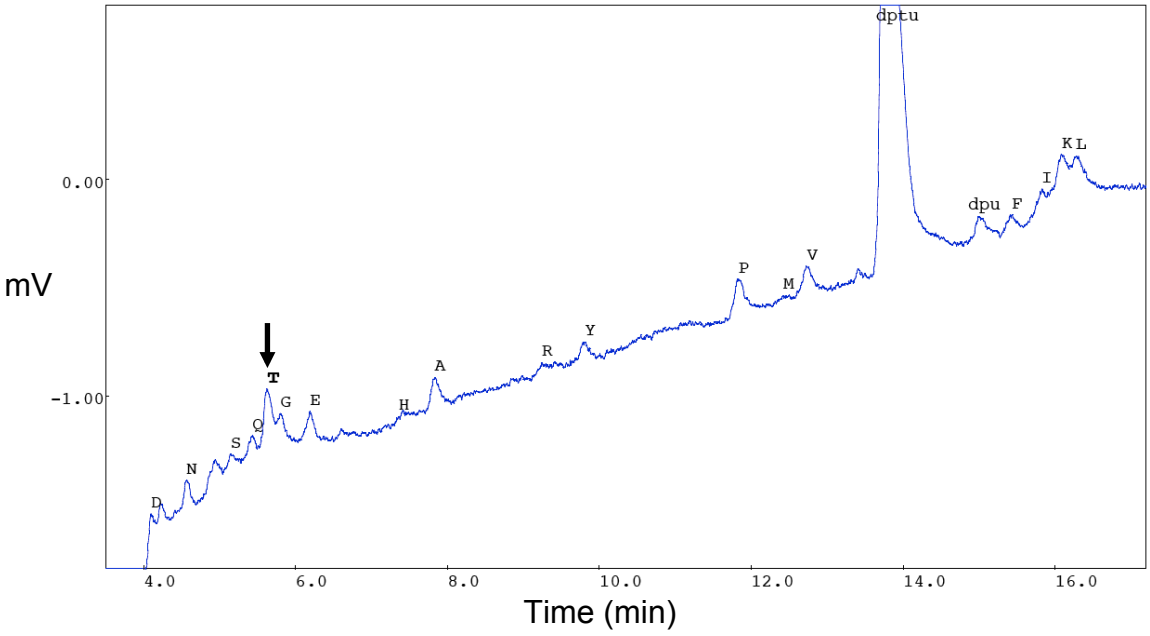


Figure 4-11. HPLC chromatogram of N-terminal amino acid residue from the 34-kDa protein in the 2M GHCl-extracted fraction of *L. gasseri* OLL2877 (residues 7 and 8).

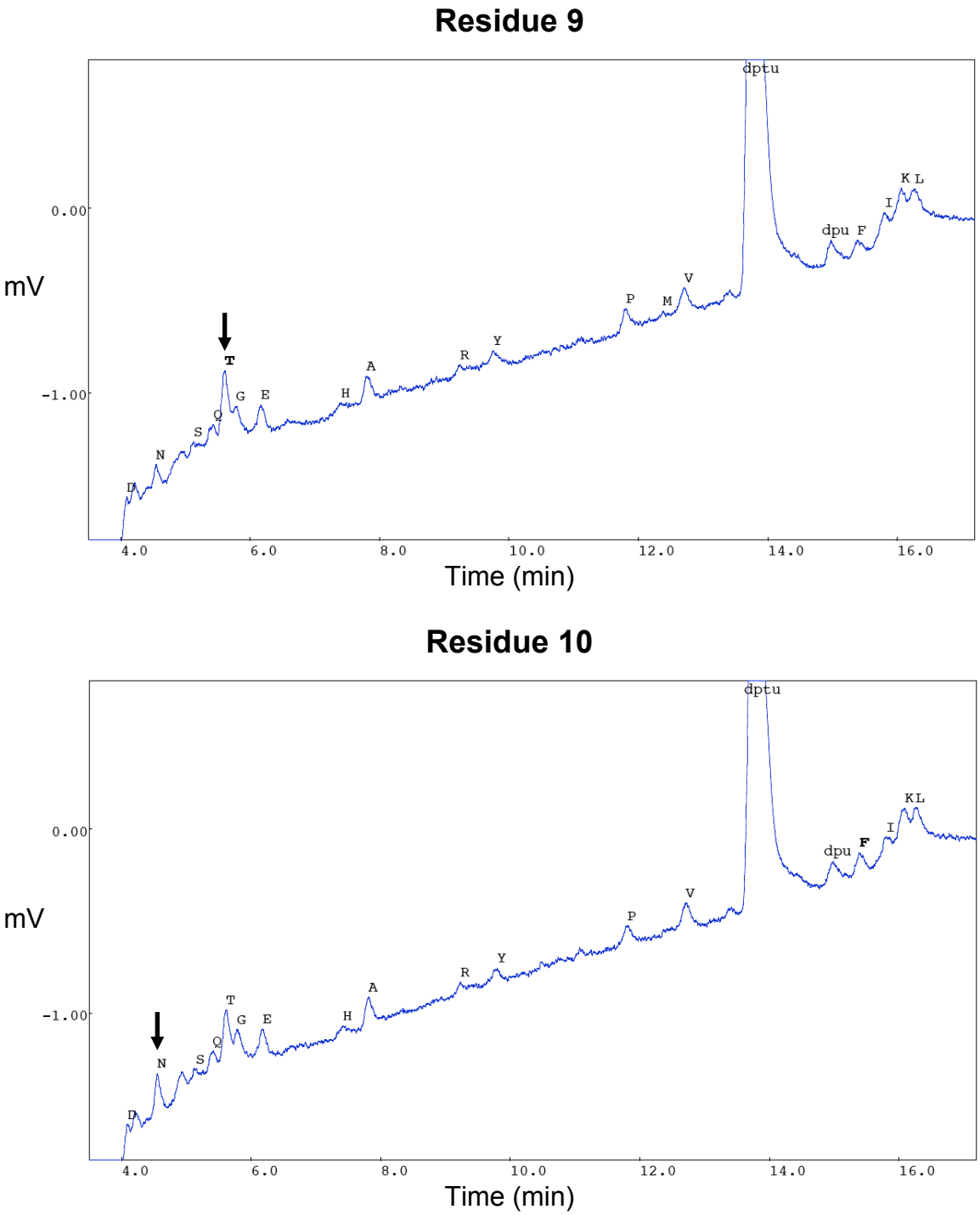


Figure 4-11. HPLC chromatogram of N-terminal amino acid residue from the 34-kDa protein in the 2M GHCl-extracted fraction of *L. gasseri* OLL2877 (residues 9 and 10).

Table 4-1. Proteins identified on the surface of *L. gasseri* OLL2877 that adhered to sulfate residue

Observed migration (kDa)	Sequence	Putative function	Theoretical migration (kDa)	Theoretical isoelectric point	Homology (%)
64	MKKTGIVMT	pyruvate kinase (*L. gasseri* JV-V03)	63.7	5.39	78
63	MKQIKFVGTL	pyruvate kinase (*L. gasseri* JV-V03)	63.7	5.39	60
34	AIIKYKPTTN	50S ribosomal protein L2 (*L. gasseri* JV-V03)	30.3	10.59	100
29	TKGILGVKVG	50S ribosomal protein L3 (*L. gasseri* JV-V03)	22.6	10.02	90
27	GQKINPEGKQ	30S ribosomal protein S3 (*L. gasseri* ATCC 33323)	24.8	10.25	88

Discussion

Bacteria adhesion to mucosal epithelial cells in host is an initial and essential factor for colonization and propagation in the gastrointestinal (GI) tract. Until now, it is known that most commensal bacteria and pathogens express adhesive molecules on their cell surfaces that interact with extracellular matrix and some specific cell receptors in the GI tract of host. Gram-negative bacteria (e.g. *Escherichia coli* and *Helicobacter pylori*) produce flagella, conjugative pili, and other cell surface adhesive components (86), whereas gram-positive bacteria (e.g. *Streptococcus*, *Staphylococcus*, and *Listeria*) produce pili, surface protein, polysaccharides and lipids that may display adhesive functions (87). These surface components mediating adherence are called adhesins. In the previous chapters, the binding between cell surface proteins of three selected strains and acidic residues have been proved. Thus, I tried to detect the surface components of the selected strains by the binding to Gal-3-S probe *in situ*, and identify the adhesin-like molecules from OLL2877, which showed the highest adhesive abilities to sHCM and acidic residues, using receptor overlay and N-terminal amino acid sequencing analyses.

The in situ detection of bacterial surface proteins showed similar results to the Biacore adhesion assay to sHCM and acidic residues that the binding of OLL2877 was significantly higher than that of OLL2877 and MCC1092 strains ($p < 0.05$). However, the Gal-3-S-binding signals were not overlaid with the signals of bacterial nucleic acid. It may indicate the bond strength of the adhesin-like components was weaker in OLL2877 than that in OLL2785, thus they were released from OLL2877. Additionally, in the preliminary experiment, the incubation of Gal-3-S probe was

performed for 1 h, but the fluorescent signal was too weak to observe under laser microscope. Thus, the incubation time was increased to overnight.

In receptor overlay analysis, results showed more binding-positive bands detected by sulfate probes than those by Neu5Ac probe. The similar patterns detected by sulfate probes in the same bacterial strain indicate that the adhesin-like molecules recognized by Gal-3-S and GlcNAc-6-S are almost identical.

Since the adhesive ability of OLL2877 was extremely higher than the other two strains, the adhesin-like components detected by sulfate residues were identified. Using N-terminal amino acid sequence analysis, the five adhesin-like components from *L. gasseri* OLL2877 were identified as pyruvate kinase (PK), 50S ribosomal protein (Rp) L2, L3, and 30S Rp S3. PK is an enzyme catalyzing the last step of glycolysis, in which pyruvate and ATP are formed (88). As the results in this study, a report indicated similar results that PK from *L. plantarum* was found as cell wall-associated protein (89). Bergonzelli *et al.* indicated that PK detected at the surface of *L. johnsonii* La1 showed strong binding to mucins obtained from HT29-MTX cells, but no stimulation of IL-8 secretion in epithelial cells and macrophage (90). Kelly *et al.* found that PK was recognized as cell-wall associated protein of *L. salivarius* subsp. *salivarius* UCC118 by two-dimensional electrophoresis, and thought to be associated with bacterial adhesion to animal and human intestinal tissue, and to both healthy and inflamed ulcerative colitis mucosa (91). Although there were few reports on bacterial PK and human intestine, PK from *Streptococcus pneumoniae* was reported as cell immunogenic component in human (92).

Rps are found in every cell, which make up the ribosomal subunits involved in the process of protein translation. They also function in multiple extraribosomal activities, including DNA repair, cell death, inflammation, tumorigenesis, and transcriptional

regulation (93). In eukaryotes, they were reported to inhibit mRNA splicing (94), shorten its own mRNA (95), promote p53 translation (96), bind to NF-κB (97), and be involved in *P. aeruginosa* pathogenesis (98). Human Rp S3 is well known to have identical amino acid sequence as a multifunctional protein involved in bacterial infection (99). On the other hand, in prokaryotes, some of Rps were reported to inhibit translation of polycistronic mRNA and S10 operon (100), participate DNA repair (101), autogenously regulate translation (102, 103), and influence RNAse E (104).

Here, Rp L2, L3, S3 were found expressing on the bacterial surface of *L. gasseri* OLL2877 by 2M GHCl extraction. A study showed that about one-third of the identified cell wall resident proteins from *Bacillus subtilis* were Rps, whereas thirteen identified Rps, including L2, L3, and S3, were found after the bacterial cells treated with trypsin (105). The localization of Rp L7/L12 was on the surface of *Streptococcus pyogenes* (106, 107), which was immunogenic in human (108). In lactic acid bacteria, Rp L2, L16, S9, and S13 were identified as surface-associated proteins in *L. rhamnosus* GG cells with 5M LiCl treatment (65). Although the possible function of Rps on bacterial surface needs further investigation, the Rps with specific binding to acidic residues were identified in this study. Rp L12 of *Neisseria gonorrhoeae* was reported to be membrane-associated and involved in invasion of human reproductive cells (109). Rp p40/laminin-binding protein appeared to be engaged in cell adhesion (110), suggesting the surface Rps may serve as adhesin when attaching to the host.

Commensal bacteria and pathogens use adhesins to attach to their host, evade immune defense, and invade to the epithelial tissue, as well as to cause disease in the host (111). After adherence, some of them also alter the mucus in the environment to increase their motility (112), degrade the mucus to use the glycoproteins as energy source (113), and disrupt the tight junction to access underlying tissues (114).

However, the adhesin studies show different approaches that have been taken to inhibit the adhesion of pathogen, in which adhesins may be therapeutic targets and vaccine candidates. Some studies designed competitive inhibitors based on detailed information from the adhesin-ligand interaction to prevent the adherence of bacteria (115, 116). A mucus-binding protein was constructed to express on the surface of lactobacilli as a marker for the diagnosis of colonic mucinous carcinomas (46). In this chapter, the adhesin-like components from the cell surface proteins of *L. gasseri* OLL2877 were identified, which showed high binding abilities to sHCM and acidic residues. Since the acidic residues are possible binding sites for enteric pathogens, OLL2877 strain would be expected for competitive exclusion of bacteria via acidic residues receptors through the five adhesin-like components in human GI tract.

CHAPTER 5

General summary

Chapter 1: Introduction

Probiotics are of significant importance to food and health industries due to their health-promoting effects when consumed. Studies have documented probiotic effects on dysbiosis, acute diarrhea, IBD, IBS, colon cancer, vaginal infection, allergy, and eradication of *Helicobacter pylori*. For the purpose of being sufficient host-interaction to confer health benefits, adhesion ability to the host gut has been considered as one of the selection criteria for probiotics strains.

The expression of acidic residues in human colonic mucin is proposed to associate with the adhesion of gut microflora. Thus, the adhesion of probiotics to acidic residues in human colonic mucin was investigated in this study.

Chapter 2: New screening methods for probiotics with adhesion properties to acidic residues in human colonic mucin using the Biacore assay

Mucin, the major protein component of the intestinal mucus layer, can be divided into neutral and acidic mucin. The acidic residues (Neu5Ac and sulfate) binding to the sugar chain of mucin are proposed to protect colon mucin against enzymatic digestion from microflora. Since increased acidic mucin and increased bacterial concentration were both observed along the human colon, the acidic residues are proposed to act as adhesion site for microflora in the intestine. To evaluate the binding of acidic mucin and bacteria, a new screening method was constructed using the Biacore adhesion assay.

The soluble human colonic mucin (sHCM) was prepared. Crude mucus scraped from the human colon tissue was digested with Proteinase K. After purified by gel filtration chromatography, the fractions containing high concentrations of sugars and

peptides were collected as sHCM, which was then immobilized on a CM5 sensor chip in the Biacore system. The removal of Neu5Ac and sulfate residues were performed using sialidase and sulfatase, respectively. Blockage of sulfate was performed using barium chloride ($BaCl_2$).

In the previous studies from my laboratory, several *Lactobacillus* and *Bifidobacteria* strains isolated from human feces were found to bind to human colonic mucin (HCM). Here I used 10 strains of lactobacilli and three strains of bifidobacteria that adhered to HCM strongly; and were selected to measure the binding to sHCM. Consequently, *Lactobacillus* strain OLL2785, *L. gasseri* OLL2877, and *Bifidobacteria bifidum* MCC1092 showed significantly higher adhesion than others ($p < 0.01$), so the three bacterial strains were selected to investigate further. After the removal of Neu5Ac from sHCM, the adhesion abilities of the three strains were decreased significantly ($p < 0.05$). However, the adhesion abilities of three strains were different after sulfatase treatment. *L. gasseri* OLL2877 showed a significant decrease after removing sulfate residues in sHCM, whereas the adhesion of OLL2785 strain and *B. bifidum* MCC1092 increased ($p < 0.05$). To further clarify the binding of sulfate residue in sHCM and bacteria, sHCM was treated with barium chloride to block the electric charge of the sulfate. Results showed a significant decrease in adhesion after barium chloride treatment ($p < 0.05$).

Accordingly, the Neu5Ac residue was recognized as a binding site by the three bacteria. OLL2785 strains and *B. bifidum* MCC1092 may have some adherence properties to sulfate, but they may have more binding abilities to Gal or GlcNAc exposed after sulfatase digestion than to the sulfate residue. Only *L. gasseri* OLL2877 showed a significant decrease after both sulfatase and barium chloride treatment, suggesting OLL2877 has a strong adhesion to the sulfate expressed in sHCM.

Chapter 3: Analysis of surface proteins from probiotics with adhesion properties to acidic residues using the Biacore assay

To further analyze the adhesive properties of bacterial surface proteins from the three selected bacteria to Neu5Ac and sulfate residues, the binding of bacterial surface proteins and the biotinylated probes with/without acidic residues were determined using the Biacore adhesion assay. Also, OLL2785 strain was identified as *L. fornicalis* in species level identification using 16S rRNA gene sequence.

The cell surface proteins from the three bacteria were collected by washing the bacterial cell body three times with PBS (PBS wash fraction) and incubating in GHCl solution (GHCl-extracted fraction). The adhesion values of untreated bacterial cells were significantly higher than that of GHCl-treated bacterial cells, suggesting the adhesin-like components were removed by GHCl solution. In both OLL2785 and OLL2877 strains, the bindings of the GHCl fraction to sHCM was significantly higher than the PBS wash fraction ($p < 0.01$), but MCC1092 showed a contrary result that the adhesion of PBS wash fraction was higher. It suggests that the adhesin-like components on bacterial surface of MCC1092 were washed off by PBS. However, the adhesin-like components from OLL2785 and OLL2877 were extracted by GHCl solution, and showed higher adhesion to sHCM than MCC1092.

The binding of GHCl fraction to the probes containing acidic probes (Gal-3-S, GlcNAc-6-S, and Neu5Ac) was also determined. The adhesion values of 2M GHCl fractions to the acidic residues were significantly higher than that of the PBS fractions ($p < 0.01$). Among different probes, the GHCl fractions of OLL785 and OLL2877 showed higher binding to sulfate residues (Gal-3-S and GlcNAc-6-S) than to Neu5Ac,

suggesting the adhesin-like components from OLL2785 and OLL2877 recognized sulfate more than Neu5Ac. However, there was no significant difference in MCC1092.

By comparing the binding to Gal-3-S/GlcNAc-6-S and the corresponding negative control Gal/GlcNAc, the effect of sulfate residue on adhesion could be clarified. Still, there was no significant difference in MCC1092. In OLL2785 and OLL2877 strains, the adhesion values of 2M GHCl fractions to acidic probes (Gal-3-S and GlcNAc-6-S) were significantly higher than to the probes without acidic residues (Gal and GlcNAc). Interestingly, though the binding to Gal-3-S was significantly higher than that to GlcNAc-6-S, the binding to Gal was lower than that to GlcNAc. It suggests that the higher binding to Gal-3-S was not contributed by the different monosaccharide linked to sulfate, but the whole structure of Gal-3-S may be recognized by adhesin-like components from OLL2785 and OLL2877.

Chapter 4: Detection and identification of adhesin-like components from probiotics with specific affinity to sulfate residue in human colonic mucin

After the binding of cell surface proteins of three selected strains and acidic residues was analyzed using the Biacore adhesion assay, I tried to detect the surface components of the selected strains by the binding to Gal-3-S probe *in situ*, and identify the adhesin-like molecules from OLL2877, which showed the highest adhesive abilities to sHCM and acidic residues, using receptor overlay and N-terminal amino acid sequencing analyses.

In the indirect fluorescent labeling of cell surface components of bacteria, the bacterial smears were fixed on slide glasses and incubated with the Gal-3-S biotinylated probe, which was detected by Alexa Fluor 488 streptavidin conjugate (green) later. The nucleic acid of bacteria was stained by DAPI. Under fluorescence

microscope, the Gal-3-S-binding components of the selected strains were observed. The Gal-3-S-binding signal was overlaid with the DNA signal OLL2785, suggesting the Gal-3-S-binding components were contained on the surface of OLL2785 cell body. However, most of the Gal-3-S-binding components were around the bacterial cell body of OLL2877. It may indicate the bond strength of the adhesin-like components was weaker in OLL2877 than in OLL2785, thus they were released from OLL2877. By analyzing the fluorescence images, the green signals were quantified and normalized by counting the particle number of DAPI signals. The fluorescent area per cell of OLL2877 was significantly higher than that of OLL2785 and MCC1092, showing similar results to the Biacore adhesion assay to sHCM and acidic residues.

Using receptor overlay analysis with different probes, the molecules with specific binding to different probes were detected. In the same bacterial strain, similar patterns were generally observed when the bands were detected by different sulfate probes, suggesting the adhesin-like molecules recognized by Gal-3-S or GlcNAc-6-S were almost identical. However, the molecules detected by Neu5Ac probe were totally different from sulfate probes, and fewer than that detected by sulfate probes.

Since the surface proteins from OLL2877 showed the highest adherence to sHCM and acidic residues, the components (at 64, 63, 34, 29, and 27 kDa) from OLL2877 specifically binding to Gal-3-S were further identified by sequencing the ten amino acids from the N-terminal end of each component. Compared to the database, the 64-kDa and 63-kDa proteins showed 78% and 60% identity with pyruvate kinase, respectively. The 34-kDa and 28-kDa proteins showed 100% and 90% identity with 50S ribosomal protein L2 and L3, respectively. Finally, the 27-kDa protein showed 88% identity with 30S ribosomal protein S3. Accordingly, the intracellular proteins were expressed on the cell surface of *L. gasseri* OLL2877, which are proposed to

contribute to the extremely higher adhesion of *L. gasseri* OLL2877 to the acidic residues linked to the sugar chain of intestinal mucin.

According to the results from this study performed for the purpose of investigating the adhesion properties of probiotics to acidic residues in human colonic mucin, the following conclusions were organized.

(1) A new screening method was developed using the Biacore assay to search for high-adhesive probiotics to Neu5Ac and sulfate residues of human colonic mucin. Three probiotics were found that recognized acidic residues.

(2) In quantitative analysis, the cell surface proteins from *L. fornicalis* OLL2785 and *L. gasseri* OLL2877 showed higher binding to sulfate residue than to Neu5Ac, and specifically recognized the whole structure of Gal-3-S.

(3) In qualitative analysis, the cell surface proteins binding to Gal-3-S were identical with the components from the same strain binding to GlcNAc-6-S, but were different from that binding to Neu5Ac.

(4) Pyruvate kinase and some ribosomal proteins expressed on the cell surface of *L. gasseri* OLL2877 are proposed to contribute to its high adhesion abilities to the acidic residues of intestinal mucin.

In this study, the binding of intestinal bacteria and the acidic residues of mucin was investigated. A new evaluation system modified from the Biacore adhesion assay is provided for the selection of probiotics, which may also help researchers understand the adhesion strategies of bowel disease pathogens and microflora in the digestive tract. The different adhesion abilities to Neu5Ac and sulfate residues showed a variation in adhesion strategy among microbes. Since the acidic residues are possible

binding sites for enteric pathogens, *L. gasseri* OLL2877 would be expected for competitive exclusion of bacteria via acidic residue receptors through the adhesin-like components in human intestine.

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